VEGF-induced angiogenesis in the ischemic brain: Effect of hyperlipidemia

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

Dr. rer. nat.

der Fakultät für

Biologie

an der

Universität Duisburg-Essen

vorgelegt von

Anil Zechariah

aus INDIEN

April 2012

Die der vorliegenden Arbeit zugrunde liegenden Experimente wurden am Institut für Zellbiologie, in der Abteilung für Vaskuläre Neurologie, Demenz- und Altersforschung am Universitätsklinikum Essen der Universität Dusiburg-Essen durchgeführt.

1. Gutachter: Herr Prof. Dr. Dirk Hermann
2. Gutachter: Frau Prof. Dr. Perihan Nalbant
3. Gutachter: Herr Prof. Dr. Erich Gulbins

Vorsitzender des Prüfungsausschusses: Frau Prof. Dr. Andrea Vortkamp

Tag der mündlichen Prüfung: 27.07.2012

LIST OF ABREVIATIONS

AAV	adeno-associated virus
Ang1	angiopoetin 1
Ang2	angiopoetin 2
ANOVA	analysis of variances
ApoE	apolipoprotein E
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BHK	baby hamster kidney
BMSC	bone marrow-derived stem cells
CBF	cerebral blood flow
CNS	central nervous system
CPS	cerebral protein synthesis
DAB	diaminobenzidine
DNA	deoxyribonucleic acid
ECA	external carotid artery
ECG	electrocardiography
ERK	extracellular-signal-regulated kinase
Epo	erythropoietin
FITC	fluorescein isothiocyanate
G-CSF	granulocyte colony-stimulating factor
HIF	hypoxia-inducible factor
HGF	hepatocyte growth factor
ICA	internal carotid artery
ICAM-1	intracellular adhesion molecule-1
IgG	immunoglobulin G
IGF	Insulin-like growth factor 1
IA	intra-arterial
ICV	intracerebroventricular
IN	intra-nasal
IP	intra-peritoneal
IV	intra-venous

LDF	Laser Doppler flow
LSD	least significant differences
MAPK	mitogen-activated protein kinase
MCA	middle cerebral artery
MIB	microvessel isolation buffer
MMP	matrix metalloproteinases
NRP-1	neuropilin-1
NO	nitric oxide
NPC	neuronal precursor cells
p75NTR	p75 neurotrophin receptor
PAI	plasminogen activator inhibitor
PCA	posterior cerebral artery
PDGF	platelet-derived growth factor
PIGF	placental growth factor
PComA	posterior communicating artery
PECAM-1	platelet and endothelial cell adhesion molecule-1 (marker for endothelial cells; CD31)
Pi3K	phosphatidylinositol 3-kinase
PPA	pterygopalatine artery
PVDF	polyvinylidene fluoride
$rhVEGF_{165}$	recombinant human VEGF165
ROI	regions of interest
RTK	receptor tyrosine kinases
SDF-1	stromal derived factor-1
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
TrkA	tropomyosin receptor kinase A
TrkB	tropomyosin receptor kinase B
TUNEL	terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

FIGURE TITELS

TABLE TITELS

Abstract

Neurovascular remodeling has been recently recognized as a promising target for neurologic therapies. Hopes have emerged that, by stimulating vessel growth, it may be possible to stabilize brain perfusion, and at the same time promote neuronal survival, brain plasticity, and neurologic recovery. However, the key question remained whether increased vessel density leads to an increase in CBF, promoting recovery. Many studies in the recent past have demonstrated the beneficial effects of therapeutic angiogenesis but had minimum benefits for patients when translated into the clinics. Evaluation of readouts which are less relevant clinically and usage of animal models which sub-optimally mimic clinical situations might be responsible for these discrepancies.

In order to understand whether the concept of induced angiogenesis is feasible from a pathophysiological point of view, we evaluated the temporal profile of capillary formation, after treating mice with VEGF and observed that new vessel formation indeed results in increased CBF and preservation of the brain energy state. Additionally, long term VEGF therapy rendered neuroprotection and better preservation of the BBB integrity. Evaluation of pericyte coverage confirmed the production of physiologically active vessels after VEGF therapy. Due to the potent, pleiotropic effects, VEGF might be a promising candidate for stimulating new vessel formation.

Hyperlipidemia affects at least half the population of stroke patients while most of the experimental investigations are done in young and healthy animals with an intact vasculature. By utilising $ApoE^{-/-}$ mice that exhibit exacerbated atherosclerosis we show that hyperlipidemia compromises new vessel formation resulting in decreased CBF and disruption of brain metabolism after focal cerebral ischemia. Additionally, hyperlipidemia increased matrix disaggregation and counteracted pericyte coverage by decreasing N-cadherin expression. Our data underlines the necessity for experimental stroke investigations utilising clinically relevant animal models that also mimic risk factors such as hyperlipidemia so as to minimise the number of study failures.

1 Introduction

1.1 Brain and its vascular system

Ever since the publication of 'De Motu Cordis' by William Harvey in 1628 there has been a growing interest into the physiological and pathological significance of the cardio vascular system. The cardio-vascular system comprising of the heart, arteries, capillaries and veins, supply the tissue with life-sustaining oxygen and other essential nutrients thereby maintaining homeostasis of the different body systems. The cardiovascular system is involved in majority of human diseases and many diseases are attributed to abnormalities in the system, as such. The human brain is an expensive organ in terms of energy usage as it uses almost one fifth of the total energy consumption in an adult human even though it corresponds to only 2% of the total body mass. As the energy reserve for the brain is only sufficient for maintaining the brain metabolic turnover for a few minutes, an uninterrupted vascular supply is warranted for a proper brain function.

Stroke is one of the most prominent disease conditions, resulting from the abolition of blood supply through the cerebral vessels and is the major cause of adult disability in the United States and Europe and the second most cause of death worldwide. Stroke occurs due the blockage of one or more of the main arteries that supply blood to the brain (ischemic stroke) or by the disruption and subsequent leakage of a blood vessel, in the brain (haemorrhagic stroke). Stroke might lead to irreversible brain damage and even death if the blood supply is not restored within a short timeframe. The vulnerability of the brain tissue and the relatively small window for therapeutic interventions makes stroke one of the most intricate disease situations to conquer.

The promotion of vascular remodeling has been recently recognized as a particularly promising therapeutic strategy in brain illnesses (Greenberg and Jin, 2005; Chen and Chopp, 2006; del Zoppo and Mabuchi, 2003; Hansen et al., 2008; Hermann and Chopp., 2012). Hopes have emerged that by stimulating the growth of new vessels, it may be possible to stabilize brain perfusion, and at the same time promote neuronal survival, plasticity, and functional recovery, once a stroke has

occurred (Chen and Chopp, 2006; del Zoppo and Mabuchi, 2003; Hermann and Chopp., 2012).

1.1.1 Physiological and pathological angiogenesis

Angiogenesis is the formation of new blood vessels from pre-existing ones. The process of angiogenesis depends on a delicate balance between stimulators and inhibitors and any divergence from this balance result in pathophysiological consequences. During the embryonic development blood vessels supply the tissues with oxygen and nutrients and in the later stages, maintain the supply to the organs by developing and integrating into a vascular network. In adults, however the process of angiogenesis is largely dormant barring conditions of wound healing, ovarian cycle and pregnancy. Induction of angiogenesis occurs when the balance between stimulators and inhibitors are tilted in favour of the former, which results from the induction of hypoxia in the tissues. Expression of hypoxia inducible factors in the tissue stimulates the production of pro-angiogenic molecules including VEGF (vascular endothelial growth factor) and angiopoeitins resulting in the stimulation, proliferation and to the formation of tubes by endothelial cells.

Angiogenesis is part of the natural defence mechanism of the brain to restore homeostasis after an ischemic accident. Angiogenesis might also play a role in neurotrophic support as the neuroblasts are found to be concentrated along blood vessels after ischemia (Yamashita et al., 2006; Beck and Plate, 2009). Indeed, stroke patients with greater microvessel density in the ischemic border show longer survival periods (Krupinski et al., 1994, Beck and Plate, 2009).

Angiogenesis is also a prominent feature of tumor growth as this provides the tumor tissue with nutrients and oxygen along with the removal of wastes. Tumor tissue release angiogenic growth factors which stimulate the outgrowth of vessels thereby enabling the cancerous tissue to undergo proliferation and metastasis. Insufficient or excessive angiogenesis is found to the common denominator for more than 70 diseases in humans including ischemia, many type of cancers, age-related blindness, cardiovascular diseases and diabetic ulcers (Folkman J et al., 2000).

Angiogenic therapy which stimulates the production of new blood vessels to restore blood supply to the starving tissues and anti-angiogenic therapy which aims to inhibit the production of new blood vessels by tumor tissue thereby inhibiting the proliferation of tumours are believed to benefit more than 500 million patients worldwide.

1.1.2 Remodelling at the neurovascular unit

Brain neurons and astrocytes critically depend on the integrity of a functional capillary system that supplies oxygen and energy-rich substrates to the tissue (Han and Suk, 2005). To safeguard this continuous supply, neurons and astrocytes release various angiogenic factors, such as VEGF, angiopoietins, bFGF (basic fibroblast growth factor), G-CSF (granulocyte colony-stimulating factor), Epo (erythropoietin), transforming growth factor-b, and insulin-like growth factor-1 (Jain and Munn, 2000; Zhang and Chopp, 2002; Park et al, 2003), which together with guidance molecules, namely semaphorins, ephrins, netrins, and their receptors (Adams and Klein, 2000; Carmeliet, 2003), induce endothelial proliferation, directed vessel growth, and formation of a functional vascular network. Similar to neurons and astrocytes, endothelial cells also release growth factors, such as brain-derived neurotrophic factor, nerve growth factor, and neurotrophins, which promote the viability of neurons, providing a sustained protection under conditions of oxygen deprivation (Carmeliet, 2003; Hennigan et al., 2007; Lok et al., 2007). At the same time, through an interaction with the receptors of these growth factors, namely Tropomyosin receptor kinase A (TrkA), Tropomyosin receptor kinase B (TrkB), and p75 neurotrophin receptor (p75NTR) (Hennigan et al., 2007), these growth factors promote reorganization processes in the brain, facilitating long-term potentiation and synaptic plasticity (Bliss and Collingridge, 1993) and promoting axon outgrowth through an interaction with guidance cues, such as semaphorins, ephrins, slits, and netrins (Bagri and Tessier-Lavigne, 2002; Dickson, 2002; Carmeliet, 2003). The mutual interactions between endothelial cells, neurons, and astrocytes have led to the concept of the neurovascular unit (Hawkins and Davis, 2005; del Zoppo and Mabuchi, 2003; Lok et al, 2007), upon which these cells should not be regarded as

separate entities but as integrated networks that need to respond to injuries in a coordinated, synergistic manner so that recovery becomes possible (Chopp et al, 2008). The physiologic interactions between neurons and vessels provide insights into endogenous protective responses activated by the brain after an ischemic attack. This raises hopes that by strengthening these mechanisms, it may be possible to promote recovery for therapeutic purposes.

1.1.3 Different types of blood vessel formation

The major processes which result in the formation of new vessels are (a) vasculogenesis (b) angiogenesis, and (c) arteriogenesis (Carmeliet, 2004). Vasculogenesis refers to the formation of blood vessels from the blood-borne or tissue-residing endothelial stem cells which migrate into the site of vascularization and differentiate to form the vascular plexus (Semenza, 2007), a phenomenon largely prominent during the embryonic stage. Angiogenesis on the other hand corresponds to the formation of capillary branches from pre-existing vessels and is the major feature of physiological angiogenesis. Arteriogenesis refers to the increase in the luminal diameter of an artery so as to increase perfusion for the tissue at risk.

The process of angiogenesis is induced by the production of hypoxia inducible factors stimulating the secretion of angiogenic growth factors. The presence of angiogenic growth factors activate the receptors present on endothelial cells resulting in the proliferation and migration of the endothelial cells. During the process of sprouting angiogenesis, the basement membrane is degraded by proteases produced by the activated endothelial cells allowing the cells to migrate to the adjacent tissue (Burri et al., 2004). The migrating cells then form sprouts, connecting vessels which then migrate to the site of the angiogenic stimulus. Sprouting angiogenesis differs from so called intussusceptive angiogenesis due to the fact that it results in the formation of completely new vessels (Burri et al., 2004). During intussusceptive angiogenesis the capillary wall of existing vessel extends into the lumen thereby slitting the vessels into two (Burri et al., 2004). Intussusceptive is prominent during the formation of embryonic vasculature as the process significantly increases the number of capillaries without the expense of endothelial cells.

1.2 Role of angiogenesis in brain ischemia

During development, central nervous system (CNS) acquires its vasculature through angiogenesis rather than vasculogenesis (Greenberg and Jin, 2005). Invading vessels from the pia matter converge towards the ventricles during the process of embryogenesis and give off secondary branches that surround the ventricles (Greenberg and Jin, 2005) resulting in the formation of cerebro-vascular system. Cerebral circulation is also distinctive from the peripheral systems by the presence of the blood brain barrier (BBB) which controls the entry of molecules to and from the brain. Disruption of the BBB during cerebrovascular accidents results in edema formation and associated consequences in the brain. Blood vessels in the brain are equipped with control mechanisms responding to the changes in local metabolic demand by effecting changes in cerebral blood flow (CBF). The structural and functional integrity of the brain depends on a continuous vascular supply of oxygen and glucose, which once interrupted causes cessation of neuronal activity leading to neuronal death from apoptotic and/or necrotic mechanisms (Hossmann, 2006). After ischemia, cerebral blood vessels in the vicinity of the stroke lesion sprout, enabling the brain parenchyma to a plethora of endogenous responses that enable successful neurological recovery (Hermann and Chopp, 2012).

1.2.1 Angiogenic responses in the ischemic brain

As angiogenesis forms a crucial part of the recovery process in many organ systems, cerebral angiogenesis has also been suggested as a natural recovery response of the brain after ischemia (Arai et al., 2009). Increased microvessel densities in the penumbra region were correlated with increased survival of human patients (Krupinski et al., 1993; Krupinski et al., 1994). On the other hand, worse prognosis was observed in patients with reduced new vessel formation after stroke (Allen, 1984; Granger et al., 1992; Szpak et al., 1999). The proliferation of endothelial cells increases exponentially after ischemia via activation of growth factors (such as VEGF) and their receptors. The synergistic regulation of angiogenesis promoters and

their receptors extends from hours to months after ischemia, suggesting the role played by angiogenesis in long-term neurovascular remodelling and recovery. Gene regulation studies in experimental stroke models have shown that endogenous signals for VEGF are expressed in neurons and astrocytes after focal cerebral ischemia (Zhang et al., 2000). Intriguingly, the receptors responsible for most of the angiogenic actions of VEGF (i.e vascular endothelial growth factor receptor2 (VEGFR2) and neuropilin) are expressed in the astrocytes only in the post ischemic state (Hermann and Zechariah, 2009).

1.2.2 Implications of angiogenesis for the ischemic brain

The responses of VEGF system and its contribution to angiogenesis lead to the question about the benefit which the brain has from increasing its vessel density. There are five major concepts on this question, which are (i) hypoxia hypothesis, (ii) capillary recruitment hypothesis (iii) neurotropic hypothesis and (iv) neuroblast migration scaffold hypothesis (v) clean-up hypothesis (summarised in figure 1).

According to the *hypoxia hypothesis*, ischemia-induced expression of hypoxia inducible factor (HIF)-1 and -2 drives the neovascularization in the infarct border zone via expression of VEGF, aiming to stabilize the perfusion of the tissue (Marti et al., 2000). Unfortunately the formation of new capillaries is slow. The density of new vessels starts to increase not before 48 hours after stroke (Seylaz et al., 1999; Pinard et al., 2000; Marti et al., 2000). VEGF delivery increases angiogenesis, but does not significantly change the time-line in which new capillaries are formed (Sun et al., 2003; Wang et al., 2005). Thus, there are few reasons to assume that angiogenesis has relevant effects on brain hemodynamics during an acute ischemic event. However, the question whether angiogenesis can protect the brain against subsequent ischemic episodes, remains to be elucidated.

The *capillary recruitment hypothesis* postulates that VEGF has a second effect besides promoting vessel growth, i.e., the improvement of vascular perfusion by relaxation of the vessels. In fact VEGF delivery acutely improved cerebral blood flow (CBF) of ischemic tissue during the first three hours after cerebral thromboembolism, when applied at high doses via systemic delivery (Zhang et al., 2000). Whether this effect reflects a restoration of vascular reactivity, e.g., by boosting endothelial NO levels, as suggested by *in vitro* studies (Wu et al., 1996), remains to be shown. It is still unclear to which extent this hemodynamic improvement contributes to VEGF's survival effect. In the experiments above (Zhang et al., 2000), no neuroprotective effect was noted after acute VEGF treatment.

According to the *neurotrophic hypothesis*, newly formed vessels are rich sources of trophic molecules that promote the survival of ischemic neurons at risk. Thus, vascular density is elevated not in order to enhance hemodynamics but to provide a microenvironment in which injured neurons are capable to survive (Chen and Chopp, 2006; Chopp et al., 2008). In contrast to the two preceding concepts, this is the first and only hypothesis that specifically relates to the brain tissue. That neurotrophic responses are important for tissue survival is supported by the dynamic responses of growth factors and their receptors, via which microvessels, neurons and glial cells interact with each other after ischemia. From a system biological view, the formation of new vessels is an energy intensive process, and it is surprising that the hemodynamically compromised brain chooses this strategy in order to survive. Thus, some open questions remain about this concept.

The *neuroblast scaffold hypothesis* assumes that newly formed vessels in the peri-infarct zone create a suitable environment for the migration, homing and differentiation of neuroblasts (Hermann and Chopp, 2012). Activated endothelial cells and vessels produces an array of trophic factors and cytokines such as stromal derived factor 1 (SDF-1), VEGF, and matrix metalloproteinases 2 and 9 (MMP2, 9) which serves as chemoattractant for neuroblast cells (Wang et al., 2006a; Chopp et al., 2007; Hermann and Chopp, 2012). Additionally, neuroblasts are found to be concentrated around blood vessels following stroke (Beck and Plate, 2009) where they migrate in close vicinity to vascular cells thus using blood vessels as a scaffold for migration in direction to the site of injury.

An alternative interpretation of the enhanced angiogenesis following stroke is the removal of dead tissue. According to the *clean-up hypothesis*, newly formed vessels allow macrophages to enter the tissue and to remove cell debris (Manoonkitiwongsa et al., 2001). In fact, the density of newly formed vessels closely correlated with that of macrophages in the ischemic brain, which supports that view (Manoonkitiwongsa et al., 2001). VEGF increased new vessel formation and macrophage infiltration in the brain (Manoonkitiwongsa et al., 2004, 2006). The clean-up hypothesis brings together two features of VEGF, its angiogenic and permeability-promoting function. As such, the main goal of newly formed vessels is not to provide oxygen and nutrients, but to guide inflammatory cells into tissue areas of damage. The question remains whether specialised vascular networks are required for the purpose of debris removal and about the validity of this concept in view of the high blood flow levels in the mammalian brain under normal, physiological conditions. Additionally, blood flow is much higher in the brain than in other tissues and the clean-up hypothesis may not only be relevant for brain tissue as such, but also for peripheral tissues but there are no convincing answers to this question.

Taken together, although angiogenesis is a key feature in the remodelling of ischemic brain tissue, the process still remains to some extent a conundrum. There is no commonly accepted explanation of why new vessel formation takes place.

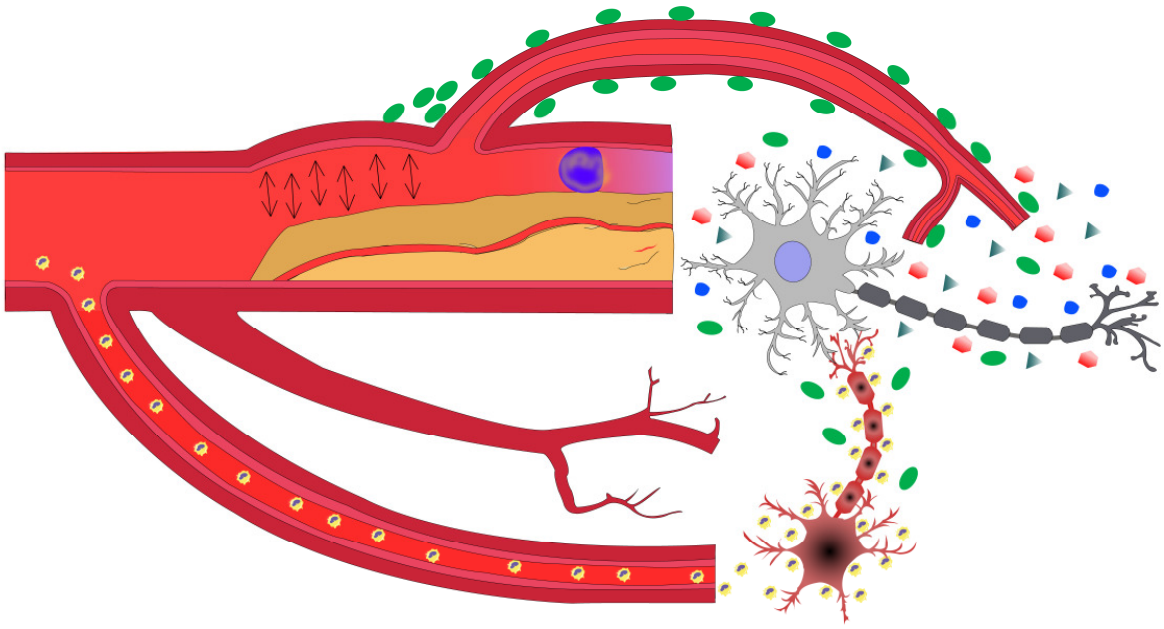


Figure 1. Five major concepts regarding the role of angiogenesis in the ischemic brain. According to the *hypoxia hypothesis*, oxygen and glucose deprivation induced by the occlusion of a brain-supplying artery (indicated by the purple coloured clot) induces vessel sprouting order to provide oxygen (indicated by blue balloons) and glucose (grey triangles) to ischemic neurons at risk. Unfortunately, the formation of new capillaries is slow. In order to overcome this limitation, VEGF also induces an acute increase of cerebral blood flow by dilation of blood vessels (indicating by bidirectional flashes in left upper Figure) according to the *vascular recruitment hypothesis*. Newly formed vessels are rich sources of trophic molecules (i.e., growth factors; indicated by red hexagons) that based on the *neurotrophic hypothesis* promote the survival of ischemic neurons at risk. As such, new vessel formation creates a microenvironment in which injured neurons are capable to survive. The *neuroblast scaffold hypothesis* suggests that newly formed vessels acts as a scaffold for neuroblasts (indicated in green colour in the upper right part of the figure) to reach the site of injury and thereby participating in the remodelling process. A further role of the enhanced angiogenesis is the removal of dead tissue. According to the *clean-up hypothesis*, newly formed vessels allow macrophages to enter the brain parenchyma and to remove cell debris (indicated in lower part of the cartoon that illustrates macrophages in close vicinity to a degenerating neuron and its axon).

1.2.3 Regulation of angiogenic growth factors after ischemia

Ischemia profoundly regulates angiogenic and neuroprotective cytokines in the brain. The expression of VEGF and its receptor (Flk-1) increase from 1 hour post stroke; an effect which is known to last at least for several days (Carmichael, 2003; Hayashi et al 2003). Neuropilin-1, co-receptor for VEGF is also expressed along the same pattern as Flk-1, as the increased expression is observed 24 hours after stroke (Carmichael, 2003; Hayashi et al 2003). The early phase of stroke also witnesses increased expression of angiopoeitin-2, Beta-3 integrin and Ephrin B2 and B4 (Carmichael, 2003; Hayashi et al 2003; Varner et al 1995).

On the other hand, the expression pattern of angiopoietin-1, which reverses the permeability effects of ang-2 is found to increase 7 days post stroke and is believed to play a role in the stabilization of new born vessels by establishing pericyte coverage (Carmichael, 2003; Hayashi et al 2003). The expression of placental growth factor is observed in the chronic phase of stroke (21 days post stroke) which corresponds to the role played by this molecule in post ischemic vascular remodelling (Carmeliet, 2000; Carmichael, 2003). On the other hand, the significance of molecules including Epo, insulin like growth factor (IGF) and hepatocyte growth factor (HGF) which has proven beneficial effects in neuronal plasticity and remodelling (Carmichael, 2003; Hayashi et al 2003) remains less clear.

1.3 The vascular endothelial growth factor

Having been discovered in the early 1980's (Senger et al., 1983) and being cloned in the late 1980's (Keck et al., 1989; Leung et al., 1989), VEGF is known for its pleotrophic actions on brain cells. Tissue protection, new vessel formation and brain plasticity are key features underlying the neurovascular remodelling induced by VEGF. All three processes are closely linked, being orchestrated in a temporospatial way. The multifaceted effects of VEGF – promoting neuronal survival, angiogenesis and brain plasticity at the same time – had nourished hopes that by use of a single molecule it may become possible to promote neurological recovery after stroke.

1.3.1 VEGF isoforms and regulation upon ischemia and hypoxia

VEGF is a pivotal regulator in angiogenesis that is markedly increased in ischemic brain tissues. As such, the growth factor well exemplifies how recovery processes are initiated and regulated in the stroke brain. In humans, the VEGF family consists of five homodimeric members (VEGF-A, VEGF-B, VEGF-C, VEGF-D, placental growth factor [PlGF]), among which VEGF-A stimulates angiogenesis in a particularly powerful way (Olsson et al., 2006; Hansen et al., 2008). VEGF-A (in the following referred to as VEGF) exists in at least six isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, $VEGF_{206}$), which differ in their binding affinity to VEGF receptors.

VEGF is ubiquitously expressed in the brain under physiological conditions mainly by choroid plexus epithelial cells, but also by astrocytes and microglia (Monacci et al., 1993; Marti and Risau, 1998). In brain hypoxia and ischemia, VEGF expression is rapidly increased within hours in a HIF-1 and HIF-2 dependent way (Forsythe et al., 1996; Marti and Risau, 1998; Abumiya et al., 1999; Lennmyr et al., 2005), and VEGF remains elevated over as long as one month after the stroke (Hai et al., 2003; Wang et al., 2004). As such, VEGF is not only upregulated on glial cells (Marti and Risau, 1998; Abumiya et al., 1999), but it is also induced on neurons (Marti and Risau, 1998), microvascular smooth muscle cells (Brogi et al., 1994; Abumiya et al., 1999), pericytes (Yonekura et al., 1999) and leukocytes (Abumiya et al., 1999). After stroke, VEGF expression is not restricted to ischemic areas, but also found in remote cortex regions (Stowe et al., 2007). Hence, additional mechanisms besides hypoxia might be involved in the regulation of this growth factor.

Angiotensin receptor antagonism in stroke animals resulted in an increase of VEGF-B and its receptor vascular endothelial growth factor receptor 1 (VEGFR1) leading to akt-phosporylation and decrease in ischemic injury (Guan et al., 2011). Vascular endothelial growth factor receptor 3 (VEGFR-3) which mediates actions of VEGF-C and VEGF-D was predominantly expressed in reactive astrocytes in the penumbra region after focal cerebral ischemia (Shin et al., 2010). Additionally, VEGF-C overexpression is able to stimulate the production of VEGFR-3-expressing NSCs and

neurogenesis in the SVZ without affecting angiogenesis (Calvo et al., 2011). The PlGF expression in cultured endothelial cells was significantly enhanced by VEGF stimulation and the upregulation of endogenous PlGF expression was significantly decreased by the inhibition of endogenous VEGF activity in vivo (Fujii et al., 2008).

1.3.2 The VEGF receptor family

VEGF binds two related receptor tyrosine kinases (RTK), VEGFR-1 (*flt*-1) and VEGFR-2 (*flk*-1, KDR), consisting of seven extracellular immunoglobulin-like domains, a transcellular domain and a consensus tyrosine kinase sequence (Shibuya et al., 1990; Terman et al., 1991). Besides VEGFR-1 and VEGFR-2, a third RTK, VEGFR-3, exists, which binds to VEGFC and VEGFD but not VEGF (Karkkainen et al., 2002). In addition to RTKs, a co-receptor, neuropilin-1 (NRP-1), has been identified (Soker et al., 1996), which is otherwise involved in axon-repulsive semaphorin-3a signalling (Neufeld et al., 2002), presenting $VEGF_{165}$ to VEGFR-2, thus enhancing VEGFR-2's signal response (Soker et al., 1998).

VEGFR-2 mediates most of the mitogenic, angiogenic and permeability-enhancing effects of VEGF (Ferrara et al., 2003; see Table 1). VEGFR-$2^{-/-}$ mice exhibit a severe lack of vasculogenesis and failure to develop blood islands, resulting in early death *in utero* (Shalaby et al., 1995). VEGFR-2 exhibits several tyrosine residues that are phosphorylated in response to VEGF (Roskoski, 2008) inducing a broad intracellular signal response involving phosphatidyl inositol-3 kinase (PI3K)/ Akt, Ras GTPase-activating protein, tyrosine kinase Src and Raf-mitogen activating protein kinase (MAPK) - extracellular regulated kinase (ERK)-1/2 pathways (Ferrara et al., 2003).

Compared with VEGFR-2, VEGFR-1 less potently influences angiogenesis and vascular permeability. VEGFR-1 was proposed to act as a decoy receptor (Park et al., 1994), representing a scavenger that prevents VEGF binding to VEGFR-2 (Roskoski, 2008). Indeed in embryonic tissues, VEGFR-1 is a negative regulator of new vessel growth (Fong et al., 1995). VEGFR-1's binding affinity to VEGF is about an order of magnitude higher than that of VEGFR-2, while its tyrosine kinase activity

is an order of magnitude lower than that of VEGFR-2 (Shibuya, 2006; Roskoski, 2008), which explains its partly antagonistic role. Being strongly expressed on macrophages, VEGFR-1 promotes inflammatory responses in peripheral tissues and perhaps atherosclerosis (Shibuya, 2006). As such, VEGFR-1's role might vary between the pathophysiological contexts, in which it is active.

Table 1: Expression of VEGF receptors in the healthy and ischemic brain

Name	Receptor type	Physiologic effect *in vivo*	Healthy brain			Ischemic brain		
			Endo-thelial cells	Neu-rons	Astro-cytes	Endo-thelial cells	Neu-rons	Astro-cytes
VEGFR-2	VEGF receptor tyrosine kinase	Mitogenic, angiogenic and permeability-enhancing activity	+	(+)	-	+	+	+
VEGFR-1	Weak VEGF receptor tyrosine kinase	Proinflammatory and atherosclerotic; ambiguous role in angiogenesis and vascular permeability	(+)	(+)	-	+	+	-
Neuropilin-1	Co-receptor to VEGFR-2; receptor for semaphorin-3a	Potentiates VEGFR-2's angiogenic and permeability-promoting activity	(+)	(+)	-	+	+	+

-, not expressed; (+), weak expression; +, moderate to strong expression

Adopted from Hermann and Zechariah (2009)

1.3.3 Role of VEGF in post-ischemic angiogenesis

In the process of angiogenesis, the loss of vascular integrity and degradation of the cell matrix are crucial initiating steps. The dilation of ischemic vessels that is evoked by release of nitric oxide (NO) (Distler et al., 2003) is rapidly followed by the loss of tight junction integrity, brought about by the phosphorylation of junctional proteins (Takenaga et al., 2009), which further goes along with the degradation of the basal lamina (del Zoppo and Mabuchi, 2003). These events are rapidly activated within only one to two hours after stroke, resulting in an increase in vascular permeability that facilitates extravasation of plasma proteins into the neighbouring tissue (Fig. 2). The extravasated plasma proteins provide mechanical support for migrating endothelial cells.

With the help of supporting cells located in the perivascular space, extracellular matrix constituents, namely laminins, collagen type IV, fibronectin, heparin sulphates and heparin sulphate proteoglycans (del Zoppo and Mabuchi, 2003) are degraded. Major effectors of this process are (i) MMPs, which already exist in proforms and are enzymatically activated upon stroke, (ii) plasminogen activator inhibitor (PAI)-1 and urokinase, which are *de novo* expressed along the infarct border on endothelial cells, astrocytes and neurons, (iii) tissue-plasminogen activator, which forms complexes with PAI-1 and (iv) angiopoietins, namely angiopoietin-1 and its natural antagonist angiopoietin-2 (see Fig. 2).

The degradation of the extracellular matrix prepares the stage for growth factors and guidance molecules. Together with other angiogenic factors, VEGF induces the proliferation of endothelial cells that migrate along a gradient of chemotactic cues (Yancopoulos et al., 2000; Ruhrberg et al., 2002; Carmeliet, 2003). The highly basic isoforms $VEGF_{189}$ and $VEGF_{206}$ are involved in this process mainly as matrix-bound short distance guidance cues, whereas $VEGF_{121}$ and $VEGF_{165}$ are more diffusible growth signals acting over larger distances (Fig. 2; Houck et al., 1992; Park et al., 1993). Endothelial migration is supported by pericytes that release several angiogenic factors, including VEGF, that migrate away from vessels into the perivascular space, where they release proteases including MMPs and urokinase, guiding endothelial cells towards their targets (Dore-Duffy and LaManna, 2007).

While migrating through the brain matrix, endothelial cells arrange themselves into cell monolayers and form tube-like structures (Fig. 2). The mesenchymal cells in the surrounding proliferate and move to the abluminal surface of the newly formed vessels. As such, endothelial cells and pericytes start to interact with each other (Hanahan, 1997; Carmeliet, 2000; Dore-Duffy and LaManna, 2007), pericytes releasing angiopoietin-1 that stabilizes the new-born vessels via its receptor Tie-2 (Fig. 2; Croll and Wiegand, 2001; Zacharek et al., 2007). The newborn vessels still need to build a functional BBB by formation of tight junctions between endothelial cells. Pericytes again control this BBB formation in an angiopoietin-1/ Tie-2 dependent way (Fig. 2; Thurston et al., 1999; Wang et al., 2007b).

It has recently been suggested that VEGF itself counteracts the maturation of new-born blood vessels by disrupting the pericyte coverage of the vessel sprouts (Fig. 2; Greenberg et al., 2008). In this study, the authors evaluated the effects of platelet-derived growth factor (PDGF), demonstrating that PDGF's receptor PDGFRβ forms a complex with one of VEGF's receptors, VEGFR-2, once PDGF is in place (Greenberg et al., 2008). Inhibition of VEGFR-2 prevented the assembly of this receptor complex and restored the maturation of the vessels (Greenberg et al., 2008).

Following hypoxia-ischemia, VEGF and angiopoietins are expressed in a temporally and spatially orchestrated fashion. As such, angiopoietin-1 is induced slightly later than VEGF in the peri-infarct borderzone, starting at 1-2 days post-stroke (Croll and Wiegand, 2001). At the same time, the expression of angiopoietin-1's counterplayer angiopoietin-2, which may have angiogenic or anti-angiogenic effects depending on its microenvironment (Jones et al., 2001; Zhu et al., 2005b), decreases (Croll and Wiegand, 2001). The temporospatially coordinated release of VEGF and angiopoietins well illustrates that the right factors need to be in place at the right time-point so that neurological recovery may become possible. Interestingly, the upregulation of VEGF takes place at a time-point at which the extracellular matrix is degraded, whereas upregulation angiopoietin-1 and downregulation of angiopoietin-2 coincide with the resolution of brain edema. Hence, molecular processes and pathophysiological changes closely go along with each other.

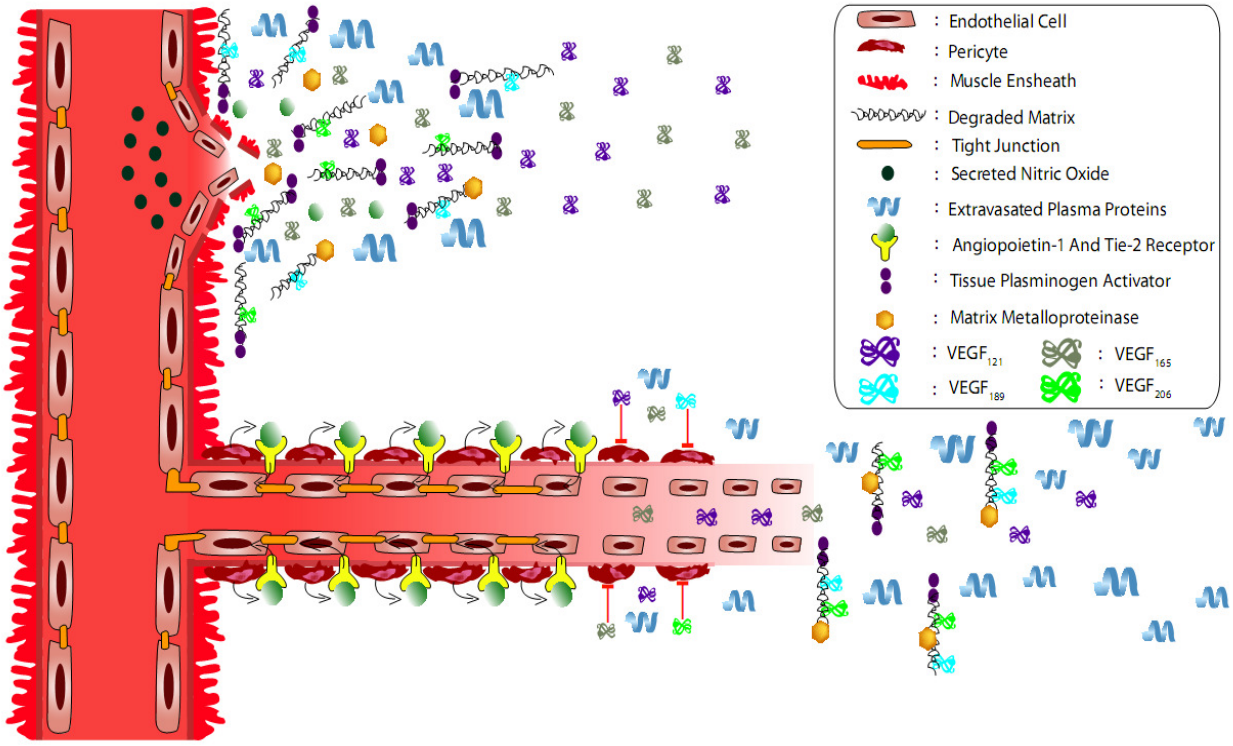


Figure 2. Upper. Role of VEGF in post-ischemic angiogenesis. In the ischemic brain tissue, the loss of vascular integrity, brought about by NO, is a crucial initiating step, which results in the extravasation of plasma proteins into the neighbouring tissue. With the help of angiopoietins, namely angiopoietin-1, MMPs and tissue-plasminogen activator, the basal membrane is degraded, which prepares the stage for VEGF that induces the proliferation of endothelial cells. The highly basic isoforms $VEGF_{189}$ and $VEGF_{206}$ (shown in blue and green) are involved in this process mainly as matrix-bound short distance guidance cues, whereas $VEGF_{121}$ and $VEGF_{165}$ (shown in purple and grey) are diffusible growth signals acting over larger distances. **Lower.** While migrating through the brain matrix, endothelial cells arrange themselves in cell monolayers and form tube-like structures. Pericytes from the surrounding proliferate and arrange around the abluminal surface of the newly formed vessels. These pericytes release angiopoietin-1, which induces the formation of tight junctions via binding to Tie-2 receptor that is localized on endothelial cells. VEGF counteracts the maturation of new-born blood vessels by disrupting the pericyte coverage of the vessels (from Hermann and Zechariah, 2009).

1.3.4 Expression of VEGF receptors in the ischemic brain

The functions of VEGF receptors are relevant not exclusively for brain microvessels but also for vessels in other tissues as well. In the adult brain, VEGFR-2 is found on newborn microvascular endothelium (Marti et al., 2000; Beck et al., 2002) that exhibit the capacity of proliferation and growth, and it is also weakly expressed on healthy neurons (Beck et al., 2002) (see Table 1). The density of VEGFR-2+ capillaries increases within 48 hours after stroke (Marti et al., 2000) and it is further elevated in the presence of VEGF (Wang et al., 2005) (Table 1). Both the level of VEGF expression and its cellular distribution, change in response to stroke. As such, VEGFR-2 more strongly appears on ischemic neurons (Jin et al., 2000; Matsuzaki et al., 2001; Wick et al., 2002; Beck et al., 2002; Kilic et al., 2006a) and astrocytes (Lennmyr et al., 1998; Kilic et al., 2006a; Choi et al., 2007) (Table 1).

Observations of an altered expression have also been made for VEGFR-1, which exhibits weak expression on endothelial cells (Beck et al., 2002) and neurons (Beck et al., 2002; Choi et al., 2007) under physiologic conditions and which is upregulated on hypoxic-ischemic endothelial cells (Lennmyr et al., 1998; Plate et al., 1999; Beck et al., 2002), neurons (Lennmyr et al., 1998; Matsuzaki et al., 2001; Wick et al., 2002) and pericytes (Levine et al., 2004; Dikov et al., 2005) during the first three days post-stroke (Table 1); for VEGFR-2's coreceptor NRP-1, which is expressed at low level on healthy neurons (Beck et al., 2002) and astrocytes (Beck et al., 2002; Choi et al., 2007) and which is elevated on ischemic endothelial cells (Beck et al., 2002), neurons (Zhang et al., 2001; Beck et al., 2002; Hou et al., 2008) and astrocytes (Zhang et al., 2001; Beck et al., 2002) over as long as 30 days post-stroke (Table 1); and for angiopoietin receptors Tie-1 and Tie-2, which are hardly found in the healthy brain (Lin et al., 2001), but more robustly expressed on ischemic cerebral vessels (Lin et al., 2000; Lin et al., 2001; Croll and Wiegand, 2001; Zhang et al., 2002) over 28 days post-stroke (Table 1).

VEGF is not the only growth factor that together with its receptors is regulated upon ischemia in the brain parenchyma. *De novo* expression on neurons and astrocytes has previously been shown also for bFGF and its receptor (Liu et al., 2006), G-CSF and its receptor (Schneider et al., 2005) and Epo receptor (Li et al.,

2007). As such, parenchymal expression appears to be a more general feature of growth factors involved in neurovascular remodeling.

1.3.5 Functionality of VEGF receptors in hypoxic-ischemic brain

That VEGF and its receptors are upregulated upon stroke should alter the responsiveness of the brain tissue. This might explain why VEGF acts not only as angiogenic but also as neuroprotective and plasticity-promoting growth factor. To exert their function and initiate cytosolic signal responses, several requirements have to be fulfilled, which relate to (i) the phosphorylation of the receptors, (ii) the interaction of VEGF receptors, namely of RTK with non-RTK, and (iii) the interaction of VEGF receptors with integrins.

Autophosphorylation of VEGF receptors. Activation of the RTK takes place upon VEGF binding via autophosphorylation (Roskoski, 2008). Two forms of autophosphorylation exist, i.e., cis-autophosphorylation, which implies that a RTK monomer catalyzes its own phosphorylation, and trans-autophosphorylation, in which two RTK monomers forming a homodimer mutually phosphorylate each other when exposed to VEGF (see Figure 2; Roskoski, 2008). Previous studies have shown that the RTK VEGFR-2 is indeed phosphorylated in the presence of VEGF after stroke and that VEGFR-2 phosphorylation goes along with downstream signal responses mediating neuronal survival and blood-brain barrier permeability (Kilic et al., 2006a).

Besides forming homodimers, RTK are probably able to form heterodimers that similar to homodimers are thought to transactivate each other by reciprocal phosphorylation. Evidence for heterodimeric RTK transphosphorylation has previously been shown in pharmacological and immunoprecipitation studies for VEGFR-1 and VEGFR-2 (Autiero et al., 2003), as well as for VEGFR-2 and VEGFR-3 (Dixelius et al., 2003). Whether transphosphorylation is relevant for VEGF receptor signaling in the brain remains unclear.

VEGF receptor crosstalk. Tyrosine kinase activity is influenced by the crosstalk of RTK with non-RTK coreceptors, namely with NRP-1, as previously shown for VEGFR-2 (Fig. 3). When interacting with VEGFR-2, NRP-1 increases VEGFR-2's binding affinity to $VEGF_{165}$ (Fig. 3; Soker et al., 1996, 1998). As a result,

a ternary receptor complex is formed (RTK/ NRP-1/ VEGF) that exhibits enhanced signal transduction properties (Kawamura et al., 2008). That RTK and NRP-1 are co-expressed upon ischemia argues in favour of a joint function of the two receptor subtypes. Observations that NRP-1 indeed promotes the activation of VEGFR-2 upon $VEGF_{165}$ treatment were recently made *in vitro* using porcine aortic endothelial cells (Kawamura et al., 2008). Evidence that this mechanism is also relevant *in vivo* after brain ischemia is still lacking.

Interactions of VEGF receptors and integrins. Integrins are receptors for cell matrix molecules, such as collagen, laminin, fibronectin and vitronectin, which control the arrangement of vascular cells and the formation of the BBB (del Zoppo and Mabuchi, 2003). Integrins exhibit rapid expression changes following stroke. As such, the collagen receptor $\alpha_1\beta_1$ strongly decreases within 2 hours after middle cerebral artery (MCA) occlusion in areas developing neuronal injury (Tagaya et al., 2001), whereas the vitronectin receptor $\alpha_v\beta_3$, which mediates endothelial and smooth muscle cell migration (Leavesley et al., 1993), was found to increase on non-capillary (>7.5 µm) microvessels (Okada et al., 1996; Abumiya et al., 1999).

Observations that $\alpha_v\beta_3$ and VEGF were co-localized after ischemia (Abumiya et al., 1999) led to protein interaction studies showing that VEGFR-2 and β_3 integrin are capable of mutually phosphorylating each other (Fig. 2; Mahabeleshwar et al., 2007). The phosphorylation of β_3 integrin was shown to be mediated by the tyrosine kinase c-Src (Mahabeleshwar et al., 2007), whereas VEGFR-2 phosphorylation was facilitated by $\alpha_v\beta_3$'s ligand, the matrix-bound sixth immunoglobin-like domain of the cell adhesion molecule L1 (Fig. 3; Hall and Hubbell, 2004). Deactivation of $\alpha_v\beta_3$ by a pharmacological inhibitor reduced VEGFR-2 phosphorylation after MCA occlusion, pointing towards the notion that $\alpha_v\beta_3$/ VEGFR-2 interaction is relevant *in vivo* (Shimamura et al., 2006).

Due to their regionally select expression that differs between capillary and non-capillary microvessels (Abumiya et al., 1999), integrins may adjust vascular migration to environmental tissue needs (del Zoppo and Mabuchi, 2003). As such, the upregulation of $\alpha_v\beta_3$ on non-capillary, but not capillary microvessels may provide an explanation for why precapillary arterioles are so important for vascular sprouting.

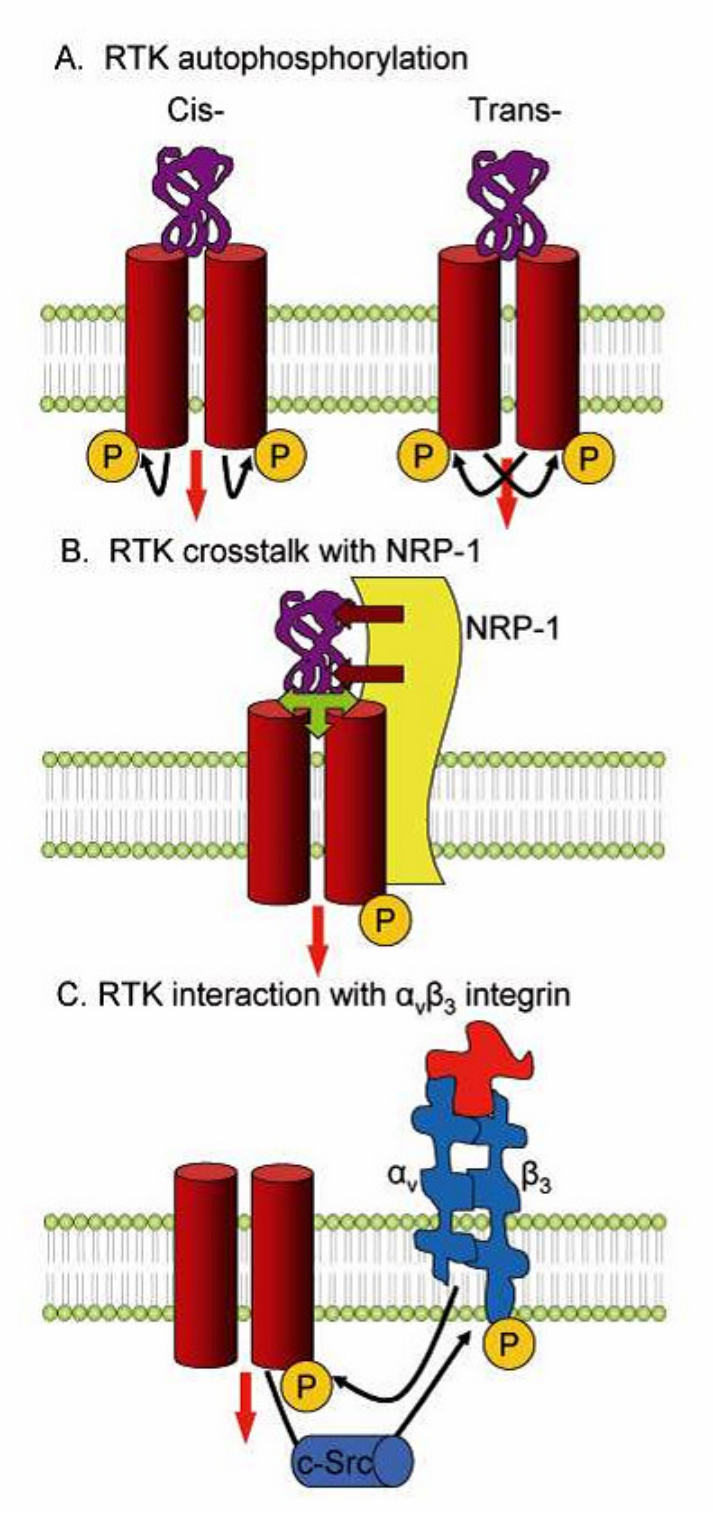


Figure 3 (for legend see page 22)

Figure 3. Mechanisms of VEGF receptor activation. Three major mechanisms have been identified on how RTK activation occurs. **A.** When bound to VEGF, RTK dimerize and subsequently get activated by autophosphorylation. Autophosphorylation may take place in a way that each RTK catalyzes its own phosphorylation (cis-autophosphorylation) or that RTK mutually phosphorylate each other (trans-autophosphorylation). **B.** The non-RTK VEGF receptor NRP-1 is capable of presenting VEGF to RTK, thereby strengthening the VEGF-RTK interaction and facilitating cell signaling. **C**. Besides binding to non-RTK VEGF receptors, RTK are capable of interacting with integrins, namely αvβ3 integrin. Upon substrate binding, αvβ3 promotes RTK phosporylation. Phosporylated RTK in turn induces the phosporylation of β3 integrin, thus generating a positive feedback loop. The latter step is mediated by tyrosine kinase c-Src (from Hermann and Zechariah, 2009).

1.3.6 Effect of other growth factors on VEGF and VEGF receptors

Besides VEGF, several other growth factors also enhance the formation of new vessels. Some of them, such as angiopoietin-1 and Epo, themselves mobilize endothelial precursor cells or recruit hematopoietic precursor cells from the blood (Losordo and Dimmeler, 2004). This process of vasculogenesis, i.e., the formation of new vessels from immature cells, has long been considered to be a feature of embryonic tissues, but is meanwhile accepted to take place also in the adult brain in parallel to angiogenesis, i.e., the growth of new vessels out of existing ones (Asahara and Kawamoto, 2004).

Not all growth factors, however, directly promote new vessel growth. In fact, some factors, including bFGF and Epo, have been suggested to exert their angiogenic function by stimulating the expression of VEGF and VEGF receptors on endothelial cells (Pepper and Mandriota, 1998; Wang et al., 2004). In case of bFGF and Epo, pharmacological inhibition of VEGFR-2 abolished the effects of the growth factors on capillary tube formation and new vessel growth (Seghezzi et al., 1998; Tille et al., 2001; Wang et al., 2004), indicating that VEGF indeed mediates their angiogenic effects. In case of Epo, *de novo* expression and secretion of VEGF on

neural progenitor cells is regulated via activation of PI3K/ Akt and ERK-1/2 pathways (Wang et al., 2008). Besides VEGF, Epo was shown to elevate bFGF, angiopoietin-2 and Tie-1 levels in the ischemic brain (Keogh et al., 2007).

VEGF-mediated vasculogenesis has previously also been shown for G-CSF, which promotes the recruitment of VEGFR-1+, VEGF-positive neutrophils as well as of circulating VEGFR-2+ endothelial progenitor cells in the blood (Ohki et al., 2005). Blockade of VEGFR-1 and to lesser extent VEGFR-2 abrogated the effect of G-CSF on new vessel growth, indicating that myelomonocytic cells play a role in the modelling of the vessels (Ohki et al., 2005). Not only growth factors but also statins increase VEGF, VEGFR-2 and brain derived neurotrophic factor (BDNF) levels in the brain, putatively by increasing subventricular zone precursor cell migration (Chen et al., 2005). Hence, VEGF appears to reflect a downstream signal in neurovascular remodelling that acts as common target of various angiogenic molecules. The combined evidence of various growth factor studies suggests that angiogenesis and vasculogenesis are closely linked in the adult ischemic brain and that VEGF plays a crucial role in both processes.

1.4 Therapeutic potential of VEGF

The effects of therapeutic VEGF delivery in the ischemic brain were analyzed in a total of thirty-four animal studies to date, which investigated actions of this growth factor on ischemic injury, blood-brain barrier disturbances, CBF and neurological recovery (summarized in Tables 2 and 3). Twenty-three of these studies were performed in rats, ten in mice and one in gerbils. Twenty-seven studies used reversible proximal (i.e., intraluminal) middle cerebral artery (MCA) occlusions. Five studies examined transcortical MCA occlusions (three permanent, two reversible), one study cerebral thromboembolism and one venous thrombosis.

Recombinant VEGF, mostly of human origin, was delivered in sixteen studies. VEGF-secreting cells were administered in three studies (neural precursor/ stem cells [NPC] in one, bone marrow-derived stem cells [BMSC] in one, baby hamster kidney [BHK] cells in one study). Adeno-associated virus (AAV) vectors were used in

four studies, plasmids containing VEGF cDNA in two studies, transgenic mouse models expressing human VEGF under a neuronal promoter in three studies and one study used transferrin coupled liposomes. Five studies evaluated VEGF antagonists.

In twenty-two studies, VEGF or VEGF antagonist was locally applied either by intracerebroventricular (ICV) or local brain delivery, or was induced by brain-specific expression. Twelve studies used systemic delivery strategies (intravenous [IV] in four, intraarterial [IA] in two, intraperitoneal [IP] in four studies and intranasal [IN] in two studies) for VEGF/ VEGF antagonist. Seventeen studies focussed on the acute injury phase, whereas sixteen studies involved post-acute assessments of functional neurological recovery up to twelve weeks post-stroke.

1.4.1 Effect on neuronal survival and blood-brain barrier integrity

Studies with therapeutic VEGF application revealed quite different effects depending on the route of growth factor delivery. As such, all twenty-one studies examining the effects of local (i.e., ICV or brain) application of VEGF, VEGF-secreting cells or vectors on structural histological injury consistently revealed a reduction of ischemic brain damage that was evaluated by infarct volumetry in twelve and analysis of disseminated neuronal injury in nine studies (see Table 2). All thirteen studies examining the impact of VEGF on functional neurological recovery exhibited sensorimotor, coordination and cognitive improvements that persisted over as long as twelve weeks post-stroke (Table 2). Along with the reduction in ischemic injury, brain edema was reduced in three studies (Zhao et al 2010., Hayashi et al., 1998; Bellomo et al., 2003) and remained unchanged in four studies (Harrigan et al., 2003; Wang et al., 2005; Kilic et al., 2006a; Miki et al., 2007), whereas a single study using encapsulated VEGF-secreting cells reported an exacerbation of edema around the grafting site (Yano et al., 2005). The extravasation of serum proteins was reduced in two studies (Kaya et al., 2005; Herz et al., 2012) and increased in two studies (Wang et al., 2005; Kilic et al., 2006a). VEGF antagonization by neutralizing antibodies reduced edema formation in another study (Chi et al., 2007).

A completely different picture resulted from the seven studies using systemic, i.e., IA, IV, IP, IN application strategies. As such, four out of six studies with systemic (IA, IV) VEGF delivery exhibited detrimental effects (Zhang et al., 2000; Abumiya et al., 2005; Kaya et al., 2005; Manoonkitiwongsa et al., 2004, 2006), reflected by an increase of brain injury in two studies, increased hemorrhagic transformation in two studies and increased microvascular injury and inflammation in one study (Table 3). One of these studies showed an increased permeability of the blood-brain barrier to gadolinium, Evans blue and fluorescein isothiocyanate (FITC) dextran (Zhang et al., 2000), one study an increase in albumin extravasation (Kaya et al., 2005) and one study no changes in immunoglobulin G (IgG) extravasation (Table 3). Two studies utilized intranasal delivery strategy during the post acute stage, resulting in reduction of infarct volume and enhanced neurological recovery (Yang et al., 2009, 2010). Four studies using VEGF antagonists revealed a reduction of infarct volume (van Bruggen et al., 1999; Kimura et al., 2005; Chiba et al., 2008; Koyama et al., 2010) and three of them showed a reduction in brain edema (van Bruggen et al., 1999; Kimura et al., 2005; Koyama et al., 2010), furthermore demonstrating that VEGF plays a detrimental role when systemically (in this case IP) applied (Table 3).

The only study exhibiting an unequivocally beneficial effect of VEGF after systemic (IA) administration used a post-acute delivery strategy, in which the growth factor was applied together with stem cells starting two days after stroke (Chu et al., 2005). This suggests that the time-point in addition to route of delivery might play a role in the actions of VEGF in the ischemic brain. Notably, all studies exhibiting unfavourable effects of VEGF used acute application strategies, in which the growth factor were delivered the day before, immediately after or one hour after the stroke (Table 3).

Table 2. Animal studies with local VEGF delivery strategies

Authors	Animal species	Stroke model	Dose	Route of delivery	Time of delivery	Angiogenesis	Brain injury	Edema/ blood brain barrier permeability	Effect on blood flow	Neurological effect	Mechanisms of action
Herz et al. (2012)	C57BL6 mouse	30 min MCA occlusion/ 3-52 days reperfusion	$rhVEGF_{165}$ (0.02 µg/day)	Local ICV	Starting 3 days after MCA occlusion	Not investigated	Reduced secondary neuronal degeneration and brain atrophy	No changes in IgG extravasation and ICAM-1+ vessels	No changes in LDF during ischemia and after reperfusion	Improved grip strength and coordination skills, associated with enhanced axonal sprouting in contralesional corticorubral tract	VEGF induces immunosuppression in both hemispheres by downregulation of broad sets of cytokines and chemokines required for the recruitment and activation of immune cells
Reitmeir et al (2012)	C57BL6 mouse	30 min MCA occlusion / 3-52 days reperfusion	$rhVEGF_{165}$ (0.004 µg/day and 0.02 µg/day)	Local ICV	Starting 3 days after MCA occlusion	Increased density of CD31+ capillaries both in ipsilesional and contralesional striatum	Reduced shrinkage of ischemic striatum, 52 days post ischemia	Not investigated	No LDF changes during ischemia and after reperfusion	Progressive improvements in grip strength and coordination skills, associated with enhanced axonal sprouting in contralesional corticobulbar tract	VEGF inhibits caspase-3 dependant apoptotic injury, post acute tissue shrinkage and enhances angiogenesis. MMP9 deactivation by VEGF resulting in lack of axonal sprouting in lesioned hemisphere. Transient downregulation of ephrin B1,ephrin B2, NG2 and brevican in contralesional hemisphere enabling axonal growth
Zhao et al. (2010)	Wistar rat	2 hrs MCAocclusion /24 hrs -7 days reperfusion	rhVEGF/ Ang1 or rhVEGF transfected adeno-associated virus (VEGF not quantified)	Local ICV	8 weeks before MCA occlusion	More strongly increased microvessel proliferation after VEGF/ Ang1 than VEGF delivery	45% reduction of infarct volume after VEGF/ Ang1 compared with VEGF delivery alone	Decreased BBB permeability following VEGF/ Ang1 compared with VEGF delivery alone	Not investigated	Marked reduction in neurological severity score following VEGF/ Ang1 compared with VEGF delivery alone	Not investigated
Wang et al. (2009)	Sprague–Dawley rat	30 min MCA occlusion / 8 weeks reperfusion	$rhVEGF_{165}$ expressing plasmids	Local ICV	15 mins after MCA occlusion	Increased number of BrdU+ and von Willebrand factor+ vessels	Reduction of infarct volume Increased number of immature and mature (also GABAergic) neurons in the cortex	Not investigated	Not investigated	Not investigated	VEGF increases neurite length and enhances maturation of stroke-induced cortical neurogenesis and dendrite formation of newborn neurons
Li et al. (2009)	Sprague–Dawley rat	2 hrs MCA occlusion/ 21 days reperfusion	$rhVEGF_{165}$ transfected adeno-associated virus (324.5 ± 86.5 pg/ml)	Local ICV	21 days before MCA occlusion	Not investigated	Reduction of infarct volume	Not investigated	Not investigated	Improved neurological function	VEGF increases the number of BrdU+ and DCX+ cells in the subventricular zone
Wang et al. (2007c)	Sprague-Dawley rat	30 min MCA occlusion/ 1 hr - 14 days reperfusion	$rhVEGF_{165}$ cDNA containing plasmids (10 µg, VEGF itself not quantified)	Local ICV	15, 120 or 360 mins after MCA occlusion	Increased endothelial proliferation indicative of angiogenesis	Reduction of infarct volume in animals treated at 15 min or 120 min, but not 360 min after MCA occlusion. Stimulation of neurogenesis in subventricular zone and striatum	Not Investigated	Not Investigated	Not Investigated	VEGF promotes neurological recovery by stimulating angiogenesis?
Shen et al. (2006)	CD1 mouse	45 min MCA occlusion/ 1-14 days reperfusion	$rhVEGF_{165}$ transfected adeno-associated virus (VEGF not quantified)	Local ICV	5 days before MCA occlusion	Not investigated	55% reduction of infarct volume, decrease of DNA fragmented cells	Not investigated	Not investigated	Not investigated	VEGF reduces caspase-3 activity
Wang et al. (2006c)	Sprague-Dawley rat	90 min MCA occlusion/ 8 wks reperfusion	$VEGF_{165}$ (0.24 µg/day)	Local ICV	24 hours after MCA occlusion	Not investigated	Not investigated	Not investigated	Not investigated	Persistently improved cognitive and sensorimotor deficits, enhanced learning and memory	Not investigated
Kaya et al. (2005)	Swiss albino mouse	90 min MCA occlusion/ 24 hrs reperfusion	$VEGF_{165}$ (15µg/kg)	Local ICV	1 hr or 3 hrs after reperfusion	Not investigated	35% and 46% reduction of infarct volume, reduced number of TUNEL positive cells	Decreased albumin extravasation	No LDF changes during ischemia and after reperfusion	Decreased motor deficits	VEGF activates PI3K/Akt pathway
Bellomo et al. (2003)	Mongolian gerbil	3-4 min bilateral CCA occlusion/ 2-12 days reperfusion	VEGF transfected adeno-associated virus (VEGF not quantified)	Local ICV	6 or 12 days before ischemia	Not investigated	Decreased neuronal injury in CA1 region	Decreased brain edema	Not investigated	Improvement in passive avoidance test	Not investigated
Harrigan et al. (2003)	Sprague-Dawley rat	2 hrs MCA occlusion/ 24 hrs reperfusion	$rhVEGF_{165}$ (0.12 µg/day)	Local ICV	1 week before MCA occlusion	Not investigated	Reduction of cortical infarct volume, no change in subcortical infarct volume	No changes in brain edema	No changes in CBF (^{14}C-isopropyl-iodoamphetamine autoradiography)	Not investigated	Reduction in infarct due to neuroprotective rather than angiogenic effects
Sun et al. (2003)	Sprague-Dawley rat	90 min MCA occlusion/ 3-28 days reperfusion	$VEGF_{165}$ (0.24 µg/day)	Local ICV	24 hrs to 3 days after MCA occlusion	Two fold increase in newly formed vWF+ blood vessels in dentate gyrus and penumbra	35% reduction of infarct volume that persisted for 4 weeks. Reduction of cell injury, cell shrinkage and DNA cleavage	Not investigated	Not investigated	Persistent decrease in neurological severity score	VEGF stimulates angiogenesis, promotes neurogenesis and reduces caspase-3-dependent neuronal death

Table 2. Animal studies with local VEGF delivery strategies (continued)

Li et al. (2008)	C57BL6 and VEGF-B deficient mouse	Transcortical MCA occlusion/(no reperfusion)/ 24hrs survival	VEGF-B (2.2 µg/µl)	Local brain (cortical)	Immediately before MCA occlusion	No effect on normal or pathological angiogenesis in the eye	50% increase of brain damage in VEGF-B deficient mice and 32% reduction in infarct volume in C57BL6 mice	Not investigated	Not investigated	Not investigated	Anti-apoptotic activity of VEGF-B is mediated by downregulation of BH3-only protein and other cell death related proteins via VEGFR-1
Chi et al. (2007)	Wistar rat	Transcortical MCA occlusion (no reperfusion)/ 1 hr survival	Anti-rhVEGF$_{165}$ antibody (25 µg and 75µg/ animal)	Local brain (cortical)	1 hr before and immediately after MCA occlusion	Not investigated	Not investigated	Transfer coefficient (K_i) of ^{14}C-α-aminoisobutyric acid reduced, indicating decreased edema	Not investigated	Not investigated	Blood brain barrier disruption during ischemia is at least in part due to endogenous VEGF already present or produced immediately after MCA occlusion
Hayashi et al. (1998)	Wistar rat	90 min MCA occlusion/ 24-48 hrs reperfusion	Gel-foam soaked rhVEGF$_{165}$ (9 ng/animal)	Local brain (cortical)	Immediately after reperfusion	Not investigated	Reduction of infarct volume and DNA fragmented cells	Decreased brain edema in VEGF treated animals	Not investigated	Not Investigated	Reduction in Hsp72 immunoreactivity indicating reduction of ischemic stress
Emerich et al. (2010)	Sprague–Dawley rat	60 min MCA occlusion/ 1 hr – 21 days reperfusion	VEGF$_{165}$ (1µg/animal)	Local brain (striatal)	15 min before MCA occlusion	Not investigated	>80% reduction of lesion volume	Not investigated	No changes in LDF during ischemia and after reperfusion	Decreased motor asymmetries and improved neurological function	Not investigated
Miki et al. (2007)	Wistar rat	2 hrs MCA occlusion/ 2-28 days reperfusion	rhVEGF$_{165}$-transfected bone marrow stem cells (BMSCs) (34 pg VEGF/g protein)	Local brain (striatal)	24 hours after MCA occlusion	Not investigated	Reduction of infarct volume	No changes in brain water content	Not investigated	Decreased motor deficits in BMSC and BMSC+VEGF treated animals, effect most pronounced in VEGF+BMSC group	Not investigated
Yano et al. (2005)	Wistar rat	90 min MCA occlusion/ 1-14 days reperfusion	Encapsulated VEGF$_{165}$ secreting baby hamster kidney (BHK) cells (11.2 ng VEGF/day)	Local brain (striatal)	6 days before MCA occlusion	Increased density of newly formed laminin+ vessels, no change in vessel diameter	48% reduction of infarct size, reduction in DNA fragmented cells	Exacerbated edema around BHK graft 6 days after capsule placement in animals receiving VEGF transfected cells	No LDF changes during ischemia and after reperfusion	Enhanced performance on accelerating rotarod at least until 14 days after MCA occlusion	VEGF activates PI3K/Akt pathway
Zhu et al. (2005a)	Sprague-Dawley rat	2 hrs MCA occlusion/ 2-12 weeks reperfusion	VEGF$_{121}$-transfected neural stem cells (VEGF not quantified)	Local brain (striatal)	3 days after MCA occlusion	Not investigated	Not investigated	Not investigated	Not investigated	Decreased motor deficits from 2 to 12 weeks in VEGF-transfected stem cell group, non-transfected stem cells without effect	Not investigated
Wang et al. (2007a)	C57BL6 mouse	Transcortical MCA occlusion (no reperfusion)/ 7-28 days survival	rhVEGF$_{165}$ under NSE promoter (21.74 ng/g protein)	Transgenic brain expression	Constitutive rhVEGF overexpression	Not investigated	Reduction of infarct volume, stimulation of neurogenesis in subventricular zone	Not investigated	Not investigated	Decreased motor deficits	VEGF promotes neurological recovery by stimulating angiogenesis?
Kilic et al. (2006a)	C57BL6 mouse	90 min MCA occlusion/ 24 hrs reperfusion	rhVEGF$_{165}$ under NSE promoter (21.74 ng/g protein)	Transgenic brain expression	Constitutive rhVEGF overexpression	Not investigated	Reduction of infarct volume that was abolished after delivery of PI3K/ Akt inhibitor Wortmannin	Increase of IgG extravasation by VEGF is completely abolished by Wortmannin	Not investigated	Decreased motor deficits reversed to the levels of wild type animals after Wortmannin treatment	VEGF activates PI3K/Akt and ERK1/2 and deactivates p38 and JNK1/2 pathways. PI3K/Akt inhibition reverses neuroprotection and blood brain barrier leakage. Neuroprotective effect mediated by VEGFR-2 that is *de novo* expressed on ischemic neurons and activated by VEGF?
Wang et al. (2005)	C57BL6 mouse	90 or 30 min MCA occlusion/ 24 and 72 hrs reperfusion	rhVEGF$_{165}$ under NSE promoter (21.74 ng/g protein)	Transgenic brain expression	Constitutive rhVEGF overexpression	Increased density of newly formed VEGFR2+ vessels in cerebral cortex and striatum	Reduction of infarct volume and DNA-fragmented cells	Increase of sodium fluorescein extravasation, no change in brain edema	58 % CBF decrease in ischemic, 59% increase in non-ischemic cortex (^{14}C-iodoantipyrine autoradiography)	Decreased motor deficits	Reduction of injury not due to hemodynamic improvement, but rather due to neuroprotective effect that involves caspase-3 inhibition

Table 3. Animal studies with systemic VEGF delivery strategies

Authors	Animal species	Stroke model	Dose	Route of delivery	Time of delivery	Angiogenesis	Brain injury	Edema/ blood brain barrier permeability	Effect on blood flow	Neurological effect	Mechanisms of action
Manoonkitiwongsa et al. (2004, 2006)	Sprague-Dawley rat	4 hrs MCA occlusion/ 7 days reperfusion	$rhVEGF_{165}$ (2, 8 or 60 µg)	Systemic IA	Immediately after reperfusion for 7 days	High VEGF dose inducing new vessel formation, low and intermediate doses without effect	High VEGF dose not influencing neuronal survival, low and intermediate doses with neuroprotective effect	High VEGF dose inducing microvascular injury, low and intermediate doses without effect	Not investigated	Not investigated	Angiogenic VEGF doses, increasing macrophage accumulation. Inflammatory changes contributing to injury following systemic VEGF delivery
Abumiya et al. (2005)	Sprague-Dawley rat	2 hrs MCA occlusion/ 1–72hrs reperfusion	rhVEGF (0.3 µg/kg)	Systemic IA	Immediately after reperfusion	No changes in vessel number	Exacerbation of hemorrhagic transformation. No change in ischemic cell injury	No changes in serum IgG extravasation	Not investigated	No changes	Not investigated
Zhao et al. (2011)	Sprague–Dawley rat	Permanent MCA occlusion (no reperfusion)/ 1-21 days survival	rhVEGF delivered via transferrin-coupled liposomes (Tf-VEGF-PL; 80 µg VEGF/ animal); control animals receiving VEGF-containing liposomes not coupled with transferrin (VEGF-PL) or normal saline	Systemic IV	48 h after MCA occlusion	Increased vascular density in penumbra of Tf-VEGF-PL treated mice compared with VEGF-PL and saline treated mice	Reduced infarct volume in Tf-VEGF-PL treated mice	Not investigated	LDF increased in Tf-VEGF-PL treated mice after 21 days	Enhanced neurological recovery in animals treated with Tf-VEGF-PL	Not investigated
Chu et al. (2005)	Sprague-Dawley rat	90 min MCA occlusion/ 35 days reperfusion	$rhVEGF_{165}$ (50 µg/kg)	Systemic IV	Neural stem cells (NSCs) injected 24 hrs, VEGF 48 hrs after MCA occlusion	Increased density of endothelial barrier antigen (EBA)+ vessels in VEGF, NSC and NSC+VEGF treated animals (latter with strongest increase)	NSC+VEGF treated groups with lesser degree of brain atrophy compared to other groups	Not investigated	Not investigated	Enhanced recovery in animals treated with NSCs and NSCs+VEGF in limb placing and rotarod tests (latter group with best results)	Not investigated
Kaya et al. (2005)	Swiss albino mouse	90 min MCA occlusion/ 24 hrs reperfusion	$VEGF_{164}$ (15 µg/kg)	Systemic IV	1 hr after reperfusion	Not investigated	Increased infarct volume	Increased albumin extravasation	No LDF changes between experimental groups during ischemia and after reperfusion	Increased motor deficits	Not investigated
Zhang et al. (2000)	Wistar rat	Thromboembolism (no reperfusion)/ 1 hr - 28 days survival	$rhVEGF_{165}$(1 mg/kg)	Systemic IV	1 or 48 hrs after thromboembolism	Increased number of plasma perfused vessels in infarct border zone	Increased hemorrhagic transformation and ischemic cell death when VEGF was given 1hr but not 48 hr after stroke	Increased extravasation of gadolinium (MRI), Evans blue and FITC dextran (histology) when VEGF was administered at 1 hr but not 48 hr after stroke	CBF increased 1-3 hrs after VEGF infusion, returned to normal at 4 hrs (perfusion weighted-MRI)	Improvements in rotarod test at 7, 14 and 28 days and in adhesive removal test at 28 days after stroke	Not investigated

Table 3. Animal studies with systemic VEGF delivery strategies (continued)

Yang et al. (2010)	Sprague–Dawley rat	90 min MCA occlusion/ 1-14 days reperfusion	$rhVEGF_{165}$ (200 µg/ml)	Systemic intranasal	Starting 3 days after MCA occlusion (administered daily until day 13)	Increased number of vWF(+) and BrdU(+)/vWF(+) cells/ increased number of FITC-dextran perfused microvessels in ischemic boundary	Reduced infarct volume	Not investigated	Not investigated	Enhanced neurological recovery	Not investigated
Yang et al. (2009)	Sprague–Dawley rat	90 min MCA occlusion/ 1-14 days reperfusion	$rhVEGF_{165}$ (30, 60 and 150 µg)	Systemic intranasal	Starting 3 days after MCA occlusion (administered on 3 consecutive days)	Increased number, segment length and diameter of microvessels after 60 and 150, but not after 30 µg VEGF	Reduced infarct volume after 60, but not 30 or 150 µg VEGF	Not investigated	Not investigated	Enhanced neurological recovery	Not investigated
Koyama et al. (2010)	Sprague–Dawley rat	2 hrs MCA occlusion/ 6 hrs - 7 days reperfusion	VEGF antagonist VGA1155 (1, 10, 25 and 50 mg/kg)	Systemic IP	30 min before MCA occlusion	Not investigated	Reduced infarct volume after VEGF antagonization	Reduced brain water content after 10 and 25 mg/kg VGA1155. VGA1155 at 10 mg/kg decreased BBB permeability to Evans blue	Not investigated	VGA1155 treatment improved neurological score, 7 days after ischemia	Not investigated
Chiba et al. (2008)	Sprague–Dawley rat	Permanent MCA occlusion (no reperfusion)/ 7 days survival	VEGF antagonist VGA1155 (10 mg/kg)	Systemic IP	30 min before MCA occlusion	Not investigated	Reduced infarct volume after VEGF antagonization	No changes in brain water content and BBB permeability for Evans blue	Not investigated	VEGF antagonization improved neurological scores, 7 days after ischemia	Not investigated
Kimura et al. (2005)	Wistar rat	Photochemical cortical venous thrombosis (no reperfusion)/ 24 hrs survival	rabbit polyclonal anti-VEGF antibody (2 mg/kg)	Systemic IP	VEGF antagonist delivered immediately after occlusion	Not investigated	Reduced venous infarct volume after VEGF antagonization	Decreased brain edema after VEGF antagonization	Not investigated	Not investigated	Reduced edema formation protecting from injury
van Bruggen et al. (1999)	C57BL6 mouse	30 or 45 min transcortical MCA occlusion/ 24hrs - 12 weeks reperfusion	VEGF antagonist mFlt (1-3)-IgG (10 mg/kg)	Systemic IP	12-16 hrs before, immediately after and one day after reperfusion	Not investigated	30% reduction of infarct volume after VEGF antagonization	Decreased brain edema after VEGF antagonization	No LDF changes between experimental groups during ischemia	Not investigated	Reduced edema formation protecting from injury

1.4.2 Effect on brain hemodynamics

One study with systemic (i.e., intravenous) VEGF delivery reported that VEGF acutely influences cerebral hemodynamics after stroke (Zhang et al., 2000). In that study, the authors used a permanent focal brain ischemia model, i.e., cerebral thromboembolism in rats, in which an exacerbation of brain edema and histological injury were noted. By means of arterial spin-labeling techniques, the authors demonstrated a short-lasting increase of CBF that persisted over as long as three hours after VEGF delivery (Zhang et al., 2000). No CBF changes were seen in non-ischemic animals. The authors concluded that the enhanced blood flow may be attributed to an enhanced vasorelaxation that was interpreted as response of the ischemic vasculature to this growth factor (Zhang et al., 2000).

In order to analyze whether hemodynamic changes are involved in VEGF's survival-promoting actions in paradigms going along with structural neuroprotection, Wang et al. (2005) studied VEGF's effects on CBF by ^{14}C-iodoantipyrine autoradiography in a model of constitutive CNS-specific VEGF overexpression. Using intraluminal MCA occlusions, the authors observed that VEGF increased regional CBF in areas outside the MCA territory, but reduced regional CBF in ischemic tissue (Wang et al., 2005). These data were interpreted as hemodynamic steal phenomenon, indicating that VEGF may redirect the blood from hemodynamically compromised to non-ischemic regions. In view of the observation that VEGF reduced infarct size in that stroke model and that neuroprotection went along in the reduction of caspase-3 levels in ischemic brain areas (Wang et al., 2005), the authors concluded that direct neuroprotective rather than hemodynamic effects are involved in the survival-promoting effects of VEGF.

1.4.3 Effect on brain plasticity and neurogenesis

A number of studies suggest that VEGF induces neurite outgrowth *in vitro* and *in vivo* (Rosenstein et al., 2003; Pitzer et al., 2003). That VEGF promotes brain plasticity is supported by the role of VEGF's receptor NRP-1 as receptor for axonal repulsion molecules belonging to the semaphorin family (He and Tessier-Lavigne., 1997). In fact, VEGF and semaphorins (in particular collapsin-1/ semaphorin-3D) compete to similar sites of the extracellular domain of NRP-1 (Miao et al., 1999). Collapsin-1 bound to neuropilin was shown to reduce lamellipodia motility in one study, resulting in cytoskeleton collapse, whereas VEGF attenuated the collapsin-1-induced lamellipodia repulsion (Miao et al., 1999). Semaphorin-3A, another repellent guidance cue, was found to induce apoptosis again through NRP-1 in another study, which was abolished by VEGF, resulting in survival, proliferation and migration of neural progenitor cells (Bagnard et al., 2001). Notably, the cellular actions of semaphorin-3A required RTK activity, semaphorin-3A's effects being blocked after VEGFR-1 inhibition. The authors interpreted their findings as further proof of evidence that NRP-1 acts as co-receptor for VEGFR-1.

Only few studies analyzed VEGF's effects on neuronal plasticity after stroke. By using ICV delivery of either recombinant VEGF or $VEGF_{165}$-containing plasmids in rats, and using constitutively VEGF-overexpressing mice, Sun et al. (2003), (Wang et al., 2009) and (Wang et al. 2007a, 2007c) reported that VEGF increased neurogenesis in the subventricular zone and striatum, increased neurite length, enhanced maturation of stroke-induced cortical neurogenesis and dendrite formation of newborn neurons (Table 2). As neurogenesis was also accompanied by improved sensorimotor recovery even when VEGF delivery was initiated as late as 24 hours after stroke (Sun et al., 2003), the authors concluded that newly formed neurons might contribute to the functional improvements. Whether and how VEGF influences the plasticity of pre-existing neurons and the remodelling of white matter tract systems is still unknown. Using anterograde tract-tracers, neutralizing antibodies directed against the growth-repulsive Nogo-A protein (Wiessner et al., 2003) as well as bone marrow-derived stem cells (Liu et al., 2007) have recently been shown to promote corticospinal sprouting contralesional to a stroke under conditions going

along with successful neurological recovery. Epo, a factor which is known to induce VEGF production in the brain, has been shown to promote neuronal survival and angiogenesis and decrease reactive astrogliosis thereby promoting perilesional tissue remodelling (Reitmeir et al., 2011). Recent investigations have shown the progressive improvements in neurological recovery and coordination skills in mice after chronic VEGF treatment, owing to the transient downregulation of ephrin b1, ephrin b2, NG2 and brevican (Reitmeir et al., 2012) and by inducing immunosuppression (Herz et al., 2012).

1.5 Angiogenesis, hyperlipidemia and stroke: Open questions

From studies in peripheral artery occlusive disease and coronary artery disease, great enthusiasm had emerged already more a decade ago (Isner et al., 1996; Hendel et al., 2000) that by therapeutic angiogenesis it might become possible to promote neurological recovery also after stroke. In coronary artery disease, experimental studies rapidly led to clinical trials, in which gene therapy approaches using intramyocardial or intracoronary delivery of VEGF cDNA plasmids or adenoviral VEGF vectors were tested. In these studies, VEGF delivery led to improvements of functionally relevant readouts, e.g., coronary vascularization or electrocardiography (ECG) abnormalities in some (Mäkinen et al., 2002; Hedman et al., 2003; Stewart et al., 2006), but not other (Rajagopalan et al., 2003; Kastrup et al., 2005) studies. In ischemic stroke, VEGF-induced permeability has precluded clinical studies in stroke until now. The question arises, whether there are applications that might nonetheless be considered for VEGF in the stroke brain. In order to answer this question, a number of open issues still have to be addressed.

Among previous studies analyzing VEGF's effects in the stroke brain, particularly few studies assessed clinically relevant stroke models, which might translate to conditions in elderly patients in which cerebral hemodynamics is compromised. Only one study assessed VEGF's effects in a clinically relevant thromboembolism model (Table 3). All studies used young animals with otherwise intact vasculature. Most importantly, no studies tested experimental conditions, in which CBF was compromised already prior to the stroke event and no studies tested

atherosclerotic or aged animals. Studies in animals with hemodynamic disturbances could mimic the clinical situation of the chronically hypoperfused brain, which is not rarely a problem in clinical neurology. Importantly, only two studies analyzed hemodynamic consequences of VEGF treatment until now (Zhang et al., 2000; Wang et al., 2005). To date, there is still no convincing evidence that therapeutic angiogenesis indeed improves blood flow. Studies in elderly animals may be of interest, since HIF-1α expression and binding activity and VEGF expression are age-dependently suppressed in smooth muscle cells, both under physiologic conditions and during hypoxia (Rivard et al., 2000).

Most studies in the past used acute treatment paradigms, delivering VEGF immediately or up to seven days following stroke (Tables 2, 3). Only four studies evaluated prophylactic treatments beginning two weeks or less prior to the stroke (Tables 2, 3). No study has so far assessed, which receptor mediates the angiogenic and neuroprotective effects of VEGF after stroke. Post-acute treatments are highly relevant with respect to future clinical developments, as based on the permeability changes it may be expected that the risk of brain edema and hemorrhagic transformation does not play a similar role in the post-acute stroke phase. Such studies should contribute additional insights into the effects of VEGF on brain plasticity processes and how VEGF promotes motor and non-motor neurological recovery. Prophylactic studies might contribute insights into the question as whether VEGF may be useful in stroke prevention, e.g., under conditions of severe intracranial stenosis.

Based on the existing literature and in view of the above-described open questions, future studies should clarify the questions, (a) whether an increased vessel density translates to improvements of CBF in the hemodynamically compromised brain, (b) how hemodynamic improvement contribute to motor and non-motor recovery, (c) which are the actions of VEGF not only in animals with preserved vasculature, where previous studies were performed, but also in hitherto animals with pre-existing atherosclerosis.

2 AIM OF THE STUDY

Major efforts have been made in human patients with atherosclerosis to stimulate angiogenesis by the delivery of vascular endothelial growth factor (VEGF) or of viral vectors or plasmids containing the VEGF transgene (Simons and Ware, 2003; Hermann and Chopp, 2012; Hermann and Zechariah, 2009; Potente et al., 2011). Functionally relevant improvements were noticed only in some (Mäkinen et al., 2002; Henry et al., 2003; Hedman et al., 2003; Stewart et al., 2006; Ruel et al., 2008; Kukula et al., 2011) but not other (Rajagopalan et al., 2003; Kastrup et al., 2005; Ripa et al., 2006) clinical studies, in which VEGF was delivered for the prevention of coronary heart or peripheral occlusive artery disease. Thus, the translation of therapeutic angiogenesis from bench to bedside still hampers.

2.1 Part I: Effect of therapeutic angiogenesis on cerebral hemodynamics, brain metabolism and recovery

In animal models of ischemic stroke, VEGF-induced angiogenesis has previously been shown to result in structural neuroprotection and functional neurological recovery (Zhang et al., 2000; Wang et al., 2005). Whether the preservation of ischemic tissue was a consequence of enhanced CBF or of the neuroprotective effects of VEGF was unclear (Hermann and Zechariah, 2009). VEGF promotes neuronal survival both directly via VEGF's receptor VEGFR2 (Kilic et al., 2006a) and indirectly by release of BDNF (Chen et al., 2005).

Although several studies have examined VEGF-induced angiogenesis in models of focal and global cerebral ischemia (Hermann and Zechariah, 2009), only two have evaluated the extent to which VEGF influences regional CBF in the ischemic brain. In a magnetic resonance imaging study, acute VEGF infusion was shown to induce a transient CBF increase in ischemic brain tissue lasting over 3 hours (Zhang et al., 2000), which was interpreted as vasorelaxation induced by this growth factor. In mice expressing human VEGF chronically under a neuron-specific

NSE promoter, increased CBF was observed in non-ischemic brain areas alongside a reduction in CBF in ischemic brain areas (Wang et al.,2005), suggesting that the enhanced angiogenesis had induced a hemodynamic steal flow. The mice examined expressed human VEGF throughout the brain, resulting in globally increased vessel densities (Wang et al.,2005). CBF was scarcely altered in this mouse line under physiological conditions (Vogel et al., 2004).

Raising further questions whether newly formed blood vessels are functional, loss of pericyte coverage of endothelial cells has previously been noticed in Matrigel assays following VEGF treatment in a model of platelet-derived growth factor (PDGF)-BB-induced angiogenesis (Greenberg et al., 2008). Immunoprecipitation studies revealed a hitherto unknown VEGF-induced deactivation of PDGF-BB's receptor PDGFRβ that is expressed on pericytes, which was mediated by the interaction of PDGFRβ with VEGF's receptor VEGFR2 (Greenberg et al., 2008). Inhibition of VEGFR2 prevented the formation of this receptor complex and restored pericyte coverage, thus stabilizing the newly formed blood vessels (Greenberg et al., 2008). Whether interactions between VEGFR2 and PDGFRβ take place *in vivo* in the brain and whether they are relevant for ischemic stroke was unknown.

In order to evaluate the concept of VEGF-induced therapeutic angiogenesis, we exposed mice that had been treated with VEGF, injected into the cerebral ventricles over a period of up to 21 days, to focal cerebral ischemia. In a multiparametric imaging strategy, combining CBF and cerebral protein synthesis (CPS) autoradiography, regional adenosine-5'-triphosphate (ATP) bioluminescence imaging and histochemical techniques, we then assessed the effects of VEGF-induced angiogenesis on regional CBF, brain metabolism and neurovascular integrity.

2.2 Part II: Effect of hyperlipidemia on VEGF-induced angiogenesis and associated benefits.

Reflecting the deposition of lipids into vessel walls either due to dietary habits or genetic predisposition, atherosclerosis is frequently accompanied by lipid abnormalities. In ischemic stroke, about half of all patients exhibit hyperlipidemia (Sacco et al., 2008; Hermann and Chopp, 2012), and hyperlipidemic subjects in turn have an elevated stroke mortality (Iso et al., 1989; Horenstein et al., 2002). Chronically increased cholesterol levels trigger a number of vascular events, such as oxidative stress, endothelial dysfunction, BBB disturbances and vascular inflammation (Duan et al., 2000; Matter et al., 2006; Osto et al., 2008; ElAli et al., 2011b).

Although the consequences of elevated cholesterol levels for vascular integrity are well-established and spontaneous angiogenesis is compromised (Van Belle et al., 1997; Duan et al., 2000; Jang et al., 2000), it is still unknown how hyperlipidemia influences angiogenesis in response to VEGF treatment after ischemia. Due to severe hemodynamic abnormalities, patients with intracranial atherosclerosis, which is particularly strongly associated with dyslipidemia (Turan et al., 2010; Park et al., 2011), might specifically be eligible for angiogenic therapies. Thus, the question how hyperlipidemic blood vessels respond to VEGF possesses high clinical relevancy.

To elucidate effects of hyperlipidemia on VEGF-induced angiogenesis, we herein treated normocholesterolemic or hypercholesterolemic C57BL6 wildtype and apolipoprotein-E (ApoE)$^{-/-}$ mice prophylactically with normal saline or VEGF for 21 days and exposed these animals to focal cerebral ischemia. We subsequently analysed changes in brain capillary density, regional CBF, energy state, histological brain injury and pericyte coverage of brain vessels.

3 MATERIALS AND METHODS

3.1 Experimental animal groups

All experiments were performed with government approval according to the National Institutes of Health guidelines for the care and use of laboratory animals.

For part 1. In the first set, 38 adult male C57BL6/j mice (20–25 grams) were randomly assigned to 6 groups that were treated with vehicle (normal saline) or recombinant human VEGF165 ($rhVEGF_{165}$) for a period of 3, 10 or 21 days (6-7 animals/ group). The MCA was then occluded for 90 min, followed by 24 hours of reperfusion. These animals were used for histochemical analysis of angiogenesis, ischemic injury and for molecular biological studies.

A second set of 12 mice were assigned to two groups (6 animals each) and treated with vehicle or $rhVEGF_{165}$ for 21 days. The MCA was then occluded for 90 min, followed by 60 min of reperfusion. These animals were used for CBF and CPS double autoradiography and regional ATP bioluminescence imaging.

For part 2. Male C57BL6/j mice and $ApoE^{-/-}$ mice were used that were fed with either regular or cholesterol-rich Western diet for six weeks starting at the age of 3 weeks. We have previously analysed plasma cholesterol levels following this diet exposure, showing that plasma cholesterol levels were 56.6±17.4 mg/dl in wildtype animals kept on regular (i.e., normal) diet, 220.4±58.0 mg/dl in wildtype animals on Western diet, 470.9±276.9 mg/dl in $ApoE^{-/-}$ animals on normal diet and 1279.1±528.2 mg/dl in $ApoE^{-/-}$ animals on Western diet in the end of the 6-week diet regime (ElAli et al., 2011b).

In a first set of studies, male wildtype mice and $ApoE^{-/-}$ mice that were fed with normal or Western diet were randomly assigned to eight groups receiving miniosmotic pump implantation into the left lateral ventricle at the age of six weeks (i.e., three weeks after the initiation of the dietary regimen), via which vehicle (0.9% NaCl) or $rhVEGF_{165}$ (0.02 µg/day) were administered over 21 days. At the age of nine

weeks, when animals had a body weight of 20-25 g, animals were exposed to 90 minutes middle cerebral artery (MCA) occlusion followed by 24 hours of reperfusion. These animals were used for histochemical analysis of angiogenesis, pericyte coverage, ischemic injury and molecular biological studies (n=6-7 animals/ group).

A second set of C57BL6/j and $ApoE^{-/-}$ mice kept on normal or Western diet were assigned to four groups treated with vehicle or $VEGF_{165}$ over 21 days in an identical way. At the age of nine weeks, animals were submitted to 90 minutes MCA occlusion followed by 60 minutes of reperfusion. These animals were used for CBF and CPS double autoradiography and for regional ATP bioluminescence imaging (6 animals/ group).

3.2 Delivery of recombinant human $VEGF_{165}$

Cannulae linked to miniosmotic pumps (Alzet 2004, Palo Alto, CA, U.S.A.) were implanted during 1.5% isoflurane anaesthesia (30% O2, remainder N2O) into the left lateral ventricle (1 mm lateral to bregma/ 2.5 mm below brain surface), for administration of normal saline or recombinant human VEGF165 (Peprotech, Hamburg, Germany; 0.02 µg/day). After implantation wounds were carefully sutured, anaesthesia was discontinued and animals were returned to their cages.

3.3 Induction of focal cerebral ischemia

Focal cerebral ischemia was induced during 1.5% isoflurane anaesthesia (30% O_2, remainder N_2O) using an intraluminal technique. Briefly, a midline neck incision was made and the left common carotid artery (CCA) and external carotid artery (ECA) were isolated and ligated. A microvascular clip (Aesculap; Tuttlingen, Germany) was temporarily placed on the internal carotid artery (ICA) and a 200µm silicon-coated nylon monofilament (Doccol Corporation, USA) was directed through the ICA, (avoiding the pterygopalatine artery (PPA)) until the origin of MCA. The monofilament was left in place for 90 minutes and then withdrawn to initiate reperfusion. Rectal temperature was maintained between 36°C and 37°C using a feedback-controlled heating system throughout the surgical procedures. Laser Doppler flow (LDF) was measured during the experiments up to 30 minutes after reperfusion using a flexible fibreoptic probe attached to the skull overlying the core of

the MCA territory. In the first set of experiments, anaesthesia was discontinued after this procedure. Wounds were sutured and animals returned to their cages. After 24 hours, animals were reanaesthetized, and sacrificed by transcardiac perfusion with 20 ml heparinised normal saline solution followed by 20 ml of normal saline. In the second set of experiments, animals remained under anaesthesia for delivery of radioactive tracers. Brains were removed and cut on a cryostat into 20 µm thick coronal sections that were retrieved at the rostrocaudal level of the bregma, which represents the level of the midstriatum. In addition, tissue samples were collected from the ischemic and contralateral non-ischemic MCA territory (striatum and overlying parietal cortex) for Western blotting, G-ELISA experiments and proteases activity assays.

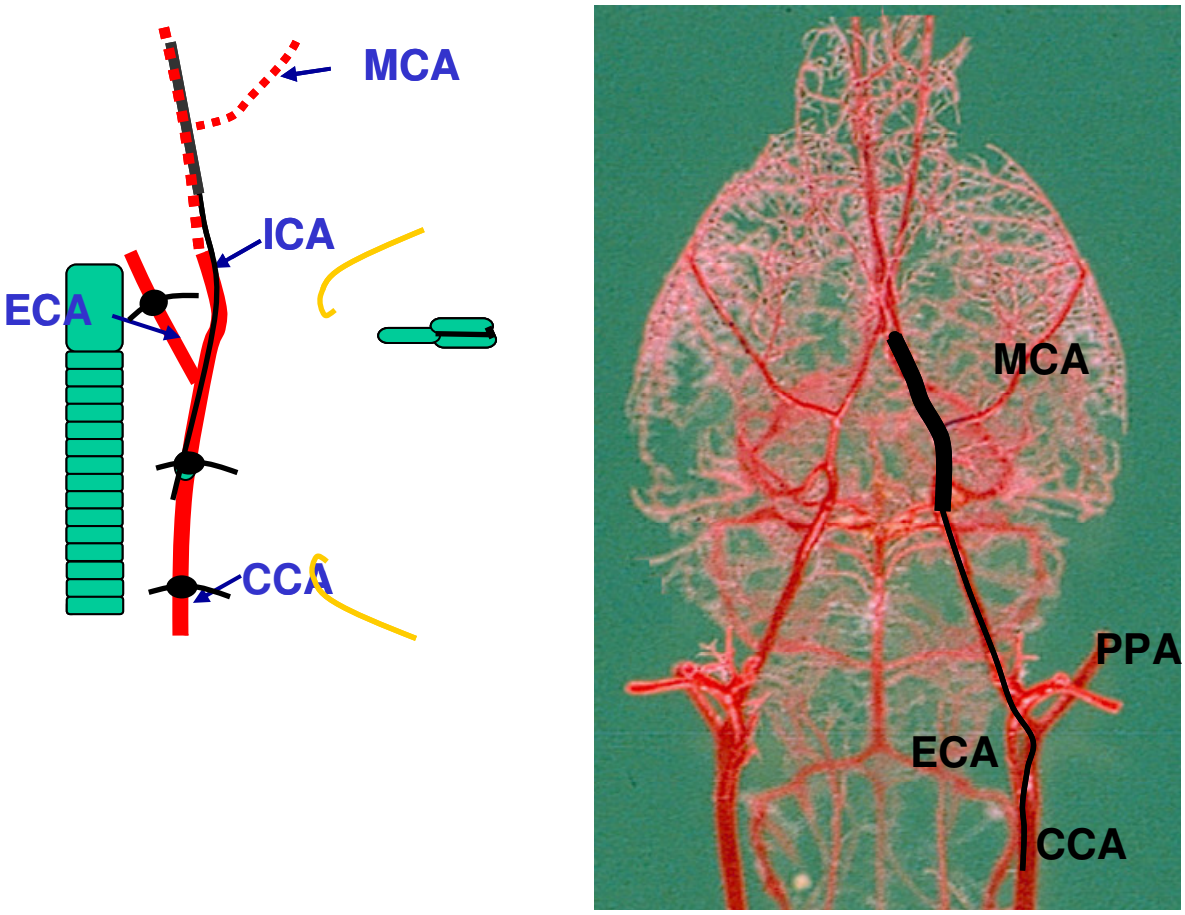


Figure 4: Middle cerebral artery occlusion model for focal cerebral ischemia. Representative pictures showing the introduction of silicon coated monofilament through the ICA impeding cerebral blood flow to the MCA supplied regions in the brain.

3.4 Evaluation of brain capillary densities

20 µm cryostat sections were prepared from brains of animals sacrificed 24 hours after reperfusion. In sections obtained from the rostrocaudal level of the mid-striatum, i.e., the site of maximum extension of the MCA territory, brain capillaries were evaluated by fluorescence immunohistochemistry using a rat anti-platelet and endothelial cell adhesion molecule-1 (PECAM-1; CD31) antibody (BD Biosciences, San Diego, CA, U.S.A.) (Kilic et al., 2006b). Stainings were evaluated by counting the number of positive vessel profiles intersecting the 10 horizontal and 10 vertical lines of a 500 µm x 500 µm grid in a fluorescent microscope (Olympus, BX41, Germany). A total of three regions of interest (ROI) in the parietal cortex and four in the lateral striatum were evaluated within the MCA territory both ipsilateral and contralateral to the stroke. Means were calculated for all ROI that were used for further analysis.

3.5 Analysis of infarct volume

Cryostat sections 1 mm apart were stained with cresyl violet (Wang et al., 2005). The border between infarcted and healthy tissue was outlined using image analysis software (Image J; National Institutes of Health) and the infarct volume was quantified by subtracting the area of intact tissue in the ischemic and contralateral non-ischemic hemisphere (Zechariah et al., 2010).

3.6 Serum IgG extravasation studies

Brain sections obtained from the mid-striatum were processed for serum IgG immunohistochemistry. 20 µm thick cryostat brain sections obtained from the midstriatum level were rinsed slightly for 5 min at room temperature in 0.1 M phosphate buffered saline (PBS), inorder to preserve endogenous intravascular IgG and was fixed with 4% paraformaldehyde / 0.1 M PBS (Kilic et al., 2006a) for 15 min at 4°C. Endogenous peroxidases were blocked by heating the brain sections with methanol/ 0.3% hydrogen peroxide (H_2O_2). Brain sections were then immersed in 0.1

M PBS containing 5% bovine serum albumin and normal goat serum (1:1000) and were incubated for 1h in biotinylated goat antimouse IgG (sc-2039; Santa Cruz Biotechnology, Nunningen, Switzerland) and stained with an avidin peroxidase kit (Vectastain Elite; Vector Labs., Burlingname, CA) and diaminobenzidine (DAB; Sigma, Deisenhofen, Germany). Brain sections were then immersed in alcohol bath and xylene, coverslipped and were used for analysis. Stained sections were scanned and converted into gray values. Sections were densitometrically analyzed by evaluating a sample of 1 x 1 mm in the core of the MCA territory (striatum and overlying cortex), from which background staining in the contralateral MCA territory was subtracted (Zechariah et al., 2010). A total of two sections were evaluated for each animal, of which mean values were calculated.

3.7 Gelatin zymography for matrix metalloproteinase (MMP)-9

Brain samples obtained from the ischemic and contralateral non-ischemic MCA territory were homogenized, lysated, supplemented with 5% protease inhibitor cocktail, and sonicated. Protein concentrations were determined using the Bradford assay. MMP-9 (gelatinase-B) activity was assessed by zymography as described (Zechariah et al., 2010). Briefly, 25 µg proteins were mixed with 5X non-reducing loading buffer for 15 min at room temperature and subjected to sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using 9% acrylamide-bis gel containing 0.1% gelatin (Sigma). Gels were removed, washed and incubated for 1 hr at room temperature with slight shaking in modified enzymatic activation buffer (50 mM Tris-HCl, 6 mM CaCl2, 1.5 µM ZnCl2, pH 7.4) containing 2.5% Triton X-100 to remove SDS and to restore gelatinase activity. Gels were then incubated for 24 hr at 37°C in modified enzymatic activation buffer. After 24 hrs gels were stained using Coomassie brilliant blue R-250 (Bio-Rad) and then were immersed in destaining solution (40% methanol, 10% acetic acid, 50% water) and 2% acetic acid. Protein extracts of all animals belonging to the same group were pooled for zymography. A total of four different gelatin gels were prepared for all experimental conditions. These gels were dried, digitized and densitometrically analyzed.

3.8 Microvessel isolation and protein extraction

Relative abundance of some proteins is much higher in the brain microvessels, compared to whole brain lysates which makes it difficult to investigate the expression changes of these proteins in whole brain homogenates. In order to overcome this issue, we isolated brain microvessels using dextran separation technique which yielded 99% pure microvessels.

Tissue samples from animals belonging to the same group were pooled and were homogenised in a glass homogenizer (Teflon®) using ice-cold microvessel isolation buffer (MIB; 15 mM HEPES, 147 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$ and 12 mM $MgCl_2$), supplemented with 5% protease inhibitor cocktail (P8340; Sigma, Deisenhofen, Germany) and 1% phosphatase inhibitor cocktail 2 (P5726; Sigma). The resulting homogenates were centrifuged at 3,200 rpm for 10 min at 4℃ and the pellets were resuspended in 20% dextran (MW 64,000-76,000; D4751, Sigma) in MIB. These suspensions were centrifuged again at 6,500 rpm for 20 min at 4℃ and the microvessel rich pellets were resuspended in MIB and filtered through two nylon filters of 100 µm and 30 µm mesh sizes (Millipore, Schwalbach, Germany). The quality of the isolated pellets was checked using the microscope and were stored at -80℃ until use. The isolated microvessels were homogenised and lysated in NP-40 lysis buffer supplemented with 5% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail, sonificated at 40% power and the protein concentrations were measured using Bradford assay kit with an iMark Microplate Reader (Bio-Rad, Hercules, Ca, USA) (ElAli and Hermann, 2011a).

3.9 Western blot analysis

Protein lysates were obtained from whole brain tissue and extracted crude microvessels using samples collected from the ischemic and contralateral non-ischemic MCA territory (ElAli, and Hermann, 2011a). The lysates were pooled, resolved by SDS-PAGE and transferred into polyvinylidene fluoride (PVDF) membranes. Membranes were immersed in blocking solution and incubated

overnight with goat anti-intercellular adhesion molecule (ICAM)-1 (BAF 796; R&D Systems, Minneapolis, MN, U.S.A.) or rabbit anti-N-cadherin (4061; Millipore, Schwalbach, Germany) antibody, diluted 1:1000. Membranes were rinsed, incubated in secondary antibody and exposed to photoluminescence solution. Protein loading was controlled using a mouse anti-β-actin antibody (4967; Millipore). Blots were repeated at least three times to confirm reproducibility, corrected to β-actin and protein levels were analyzed densitometrically.

3.10 Analysis of Rac1 Rho GTPase by G-LISA™

Rac1 activity was analyzed by G-LISA™ Rac activation assay (BK125; Cytoskeleton, Frankfurt, Germany) using protein lysates from cerebral microvessels, according to the manufacturer's protocol and as described (ElAli, and Hermann, 2011a). Absorbance was measured at 490 nm using the iMark microplate reader (Bio-Rad, Hercules, CA, U.S.A.).

3.11 CBF and CPS double-autoradiography

Fifteen minutes after reperfusion, 150 µCi L-[4, 5-3H] leucine (specific activity 151 Ci/mmol; Amersham, Braunschweig, Germany) was administered intraperitoneally, followed by an intraperitoneal injection of 10 µCi 4-iodo-N-methyl-[14C] antipyrine (Amersham) 43 minutes later. After a further two minutes, animals were instantly frozen in liquid nitrogen. Blood samples were obtained from the heart, in which the activity of 4-iodo-N-methyl-[14C] antipyrine was measured. Brains were removed and cut into 20 µm thick sections that were mounted on poly-l-lysine coated slides. These were exposed for 14 days, together with ^{14}C and ^{3}H standards on ^{14}C-Hyperfilm (Amersham) for CBF autoradiography. Brain slices were then incubated for 24 h in 10% trichloroacetic acid to remove labeled free leucine and metabolites other than proteins, and subsequently re-exposed for the same duration to perform ^{3}H autoradiography of ^{3}H-labeled proteins using Hyperfilm 3H (Amersham) (Maeda et al., 2000; Hermann et al., 2001). Regional CBF was calculated as described previously (Wang et al., 2005; Maeda et al., 2000) by calibration with the ^{14}C- and ^{3}H-standards, and radioactivity values measured in the blood. In the calibrated sections, regional CBF was determined in four ROI in the ischemic lateral striatum and three

ROI in the overlying parietal cortex (all inside MCA territory). For each animal, mean values were formed for all ROI.

3.12 Regional ATP bioluminescence imaging

For ATP measurement, frozen sections were freeze-dried and coated with a layer of frozen reaction mix containing the enzymes, coenzymes and cofactors necessary for evoking ATP-specific bioluminescence (Kogure and Alonso, 1978; Mies et al., 1991). The tissue/enzyme bilayer was thawed and light emissions were recorded using a CCD camera.

3.13 Evaluation of metabolic penumbra

The CPS-deficient and ATP-depleted area was determined on the CPS autoradiography and ATP bioluminescence images by outlining areas with preserved CPS and ATP in both hemispheres at the level of the mid-striatum. The metabolic penumbra was calculated from these results by subtracting the ATP preserved area from the CPS deficient area (Mies et al., 1991).

3.14 Evaluation of pericyte coverage of brain capillaries

Cryostat sections from the level of the mid-striatum were processed for double immunohistochemistry using rabbit anti-desmin (Abcam, Cambridge, UK) and rat anti-CD31 (BD Biosciences) (Greenberg et al., 2008; Helfrich et al., 2010).

To evaluate pericyte coverage of brain capillaries, the percentage of pericyte positive microvessels was counted in seven ROI's (three in the parietal cortex and four in the lateral striatum) ipsilateral and contralateral to the stroke, out of which mean values were formed.

To evaluate further, the volume changes in pericyte coverage, confocal 3D stacks were obtained using laser scanning microscope (LSM 510; Carl Zeiss MicroImaging, Jena, Germany) using 20 µm sections scanned at 2 µm intervals,

which were viewed using Zeiss LSM image browser and analyzed using ImageJ software. In these stacks, CD31+ capillaries and desmin+ pericytes were outlined, allowing for the evaluation of capillary and pericyte volumes, respectively, which were integrated for all levels, resulting in capillary and pericyte volumes, from which volume ratios were formed.

3.15 Evaluation of pericytic injury and expression of VEGF receptors

Cryostat sections from the level of the mid-striatum were processed for double immunohistochemistry using rabbit anti-desmin (Abcam, Cambridge, UK), rabbit anti-cleaved caspase-3 (AB3623, Millipore), goat anti-VEGFR2 (AF644; R&D Systems) and rat anti-VEGFR1 (MAB471; R&D Systems) antibodies (Greenberg et al., 2008; Helfrich et al., 2010). To evaluate DNA fragmentation in pericytes, terminal transferase biotinylated-dUTP nick end-labeling (TUNEL) was performed using the fluorescein in situ cell death detection kit (11684795910; Roche, Basel, Switzerland), followed by immunohistochemistry for desmin. Brain sections doublestained for desmin, VEGR2, VEGFR1 and cleaved caspase-3 were used to evaluate the presence of VEGF receptors on pericytes and to test, if ischemic pericytes express activated caspase-3. In sections processed for TUNEL and desmin DNA fragmentation was evaluated. For the latter analyses, seven ROI were evaluated (three in the parietal cortex and four in the lateral striatum), with the investigator being blinded for experimental conditions at all stages of the data analysis.

3.16 Statistics

LDF recordings were evaluated by repeated measurement analysis of variance (ANOVA) with values determined at 15 min intervals during MCA occlusion and at 5 min intervals after reperfusion.

For part 1. Capillary density, infarct volume, IgG extravasation, and all other histochemical studies comparing four or six groups were evaluated by two-way ANOVA, of which significant results are reported in the text. For those variables exhibiting significant differences, two-tailed t-tests were computed as post-hoc tests.

Changes in CBF, ATP depletion and the metabolic penumbra were analyzed by two-tailed t-tests, as were histochemical studies with comparisons between two groups.

For part 2. As repeated measurement ANOVA for the LDF recordings revealed significant group x time interaction effects, oneway ANOVA were computed for various time points, for which in case of significance, least significant difference (LSD) tests were calculated as post-hoc tests. Infarct volume, capillary density, IgG extravasation, MMP-9 activity and pericyte coverage were evaluated by oneway ANOVA followed by LSD tests. Three-way were also computed, from which significant interaction effects are reported in the text. Regional CBF, the metabolic penumbra and the area exhibiting ATP depletion were evaluated by oneway ANOVA followed by LSD tests. Two-way ANOVA were also calculated, from which significant interaction effects are presented. All data are presented as means ± SD. $P \leq 0.05$ was considered significant.

4 Results

4.1 Effect of VEGF on brain angiogenesis, cerebral hemodynamics and tissue injury

4.1.1 VEGF increases capillary density in a time dependant manner

To assess how the i.c.v. delivery of VEGF influences microvascular networks in a time-dependent manner, capillary densities were analyzed by CD31 immunohistochemistry. In comparison with vehicle treated animals, VEGF significantly increased capillary density from the 10th day post treatment in the ischemic hemisphere (Fig. 5A), where VEGF was applied and which for this reason was expected to exhibit the highest VEGF tissue concentrations. The effect persisted for up to 21 days after initiation of VEGF treatment (Fig. 5A). Two-way ANOVA revealed significant VEGF ($F_{1,30}$=25.116; p< 0.001) and VEGF x treatment duration interaction ($F_{2,30}$=3.426; p< 0.05) effects. In the contralateral hemisphere, at distance to the VEGF infusions, capillary density did not differ between groups (Fig. 5B).

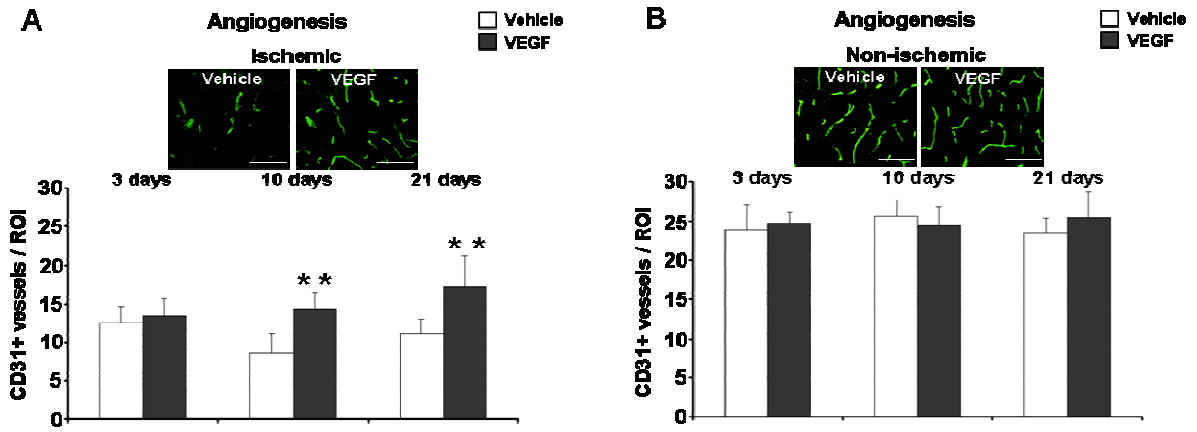


Figure 5. VEGF enhances cerebral capillary formation. CD31 immunohistochemisty showing increased brain capillary densities starting 10 days after initiation of VEGF treatment (A) in the ischemic hemisphere of animals, to which VEGF was delivered, but not (B) the contralateral non-ischemic hemisphere at distance to the infusion site. Representative photographs from animals treated with vehicle or VEGF for 21 days are shown. Data are means ± SD (n=6-7 animals/ group). *p< 0.05/ **p< 0.01 compared with vehicle. Bar, 100 µm.

4.1.2 VEGF induces neuroprotection after ischemia

To study the influence of VEGF on histological signs of brain injury after focal cerebral ischemia, mice were exposed to 90 min MCA occlusion. LDF recordings taken during and after MCA occlusion to control the reproducibility of ischemias did not reveal any differences between groups (Fig. 6A). Upon MCA occlusion, LDF decreased to 10-20% of baseline values in all groups. Reperfusion was associated with a rapid restoration of blood flow.

Infarct measurements on cresyl violet staining 24 hours after reperfusion revealed reduced infarct volumes in animals treated with VEGF for 10 or 21, but not for 3 days (Fig. 6B). Two-way ANOVA demonstrated robust effects of VEGF on infarct volume ($F_{1,30}=9.846$; $p< 0.01$).

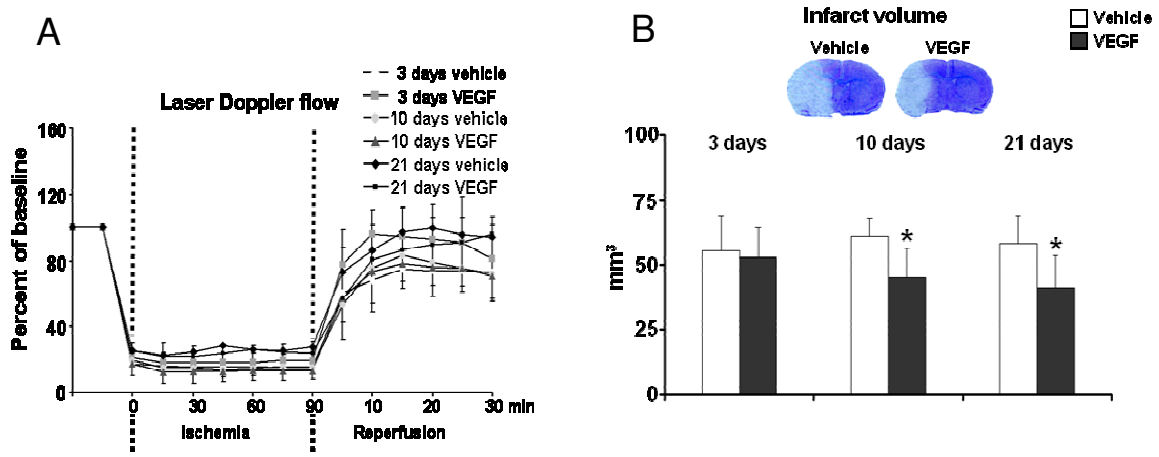


Figure 6. VEGF attenuates brain injury following subsequent focal cerebral ischemia. Laser Doppler flow recordings during and after 90 minutes MCA occlusion. Note the reproducibility of MCA occlusions and reperfusions. No differences between groups were found (Fig 6A). Infarct volumes exhibiting reduction of ischemic injury after VEGF treatment for 10 or 21, but not 3 days (Fig 6B). Representative photographs from animals treated with vehicle or VEGF for 21 days are shown. Data are means ± SD (n=6-7 animals/ group). *$p< 0.05$/ **$p< 0.01$ compared with vehicle. Bar, 100 µm.

4.1.3 Long term VEGF treatment preserves BBB integrity

The attenuation of brain injury was associated with reduced IgG extravasation in animals receiving VEGF for 21, but not for 3 or 10 days (Fig. 7), demonstrating the formation of intact vessels are not associated with blood-brain barrier leakage. Two-way ANOVA demonstrated robust effects of VEGF on IgG extravasation ($F_{1,30}$=5.028; p< 0.05).

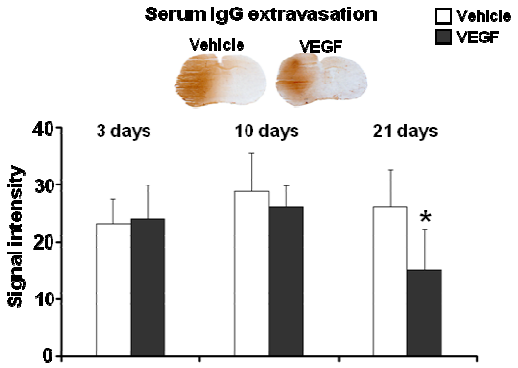


Figure 7. VEGF reduces post-ischemic serum IgG extravasation. IgG immunohistochemistry demonstrating reduced IgG extravasation after VEGF treatment for 21, but not 3 or 10 days. Representative photographs from animals treated with vehicle or VEGF for 21 days are shown. Data are means ± SD (n=6-7 animals/ group). *p< 0.05/ **p< 0.01 compared with vehicle.

4.1.4 VEGF reduces extracellular matrix disaggregation and ICAM1 expression

To assess how VEGF influences extracellular matrix integrity and neuroinflammation, MMP-9 activity and ICAM-1 expression were analyzed by gelatin zymography and Western blots, respectively. Reduced MMP-9 activity was noted in ischemic tissues of animals receiving VEGF treatment for all treatment durations examined (Fig. 8 A), thus demonstrating that VEGF-induced angiogenesis is not achieved at the expense of excessive extracellular matrix breakdown. Two-way ANOVA revealed an effect of VEGF on MMP-9 activity ($F_{1,18}$=40.185; p< 0.001). ICAM-1 expression in whole-brain lysates was transiently reduced by VEGF at 10 days post treatment (Fig. 8 B). Two way ANOVA revealed a significant effect of VEGF on ICAM-1 ($F_{1,24}$=4.517; p< 0.05).

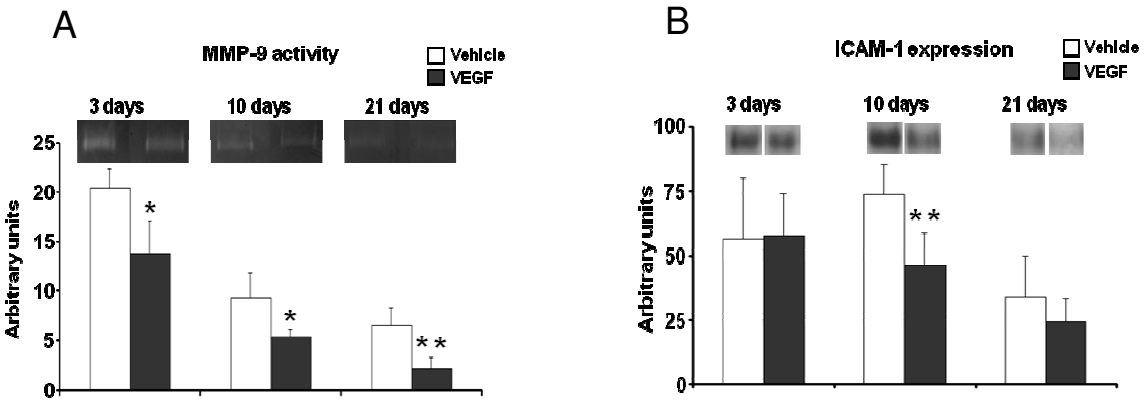


Figure 8. VEGF reduces MMP-9 activity and ICAM-1 expression in the ischemic brain. (**A**) Gelatin zymography showing decreased MMP-9 activity after 3, 10 and 21 days of VEGF treatment, demonstrating that VEGF-induced angiogenesis is not achieved at the expense of increased extracellular matrix breakdown. (**B**) Western blots showing reduced ICAM-1 expression after 10 days of VEGF delivery, revealing an anti-inflammatory effect. Data are means ± SD (n=≥3 independent zymographies or blots. *p< 0.05/ **p< 0.01 compared with vehicle.

4.1.5 Rac1 activity is increased after VEGF treatment

The RhoGTPase Rac1 induces endothelial motility and proliferation, actin cytoskeleton polymerization and endothelial barrier formation (Sawada et al., 2008), and can thus be used as a marker of angiogenesis. Rac1 G-LISA revealed that whereas no change in Rac1 activity was found in capillary extracts of animals treated with VEGF for 3 or 10 days, increased Rac1 activity was observed in ischemic tissues exposed to VEGF for 21 days (Fig. 9). Two-way ANOVA revealed a VEGF x treatment duration interaction effect for Rac1 activity ($F_{2,32}$=4.506; p< 0.05).

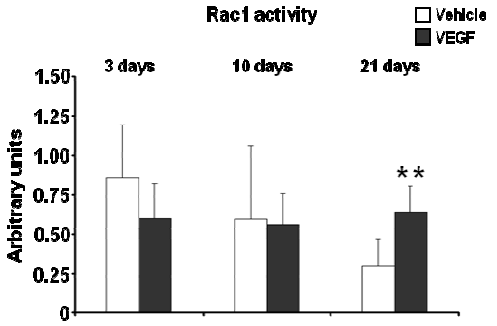
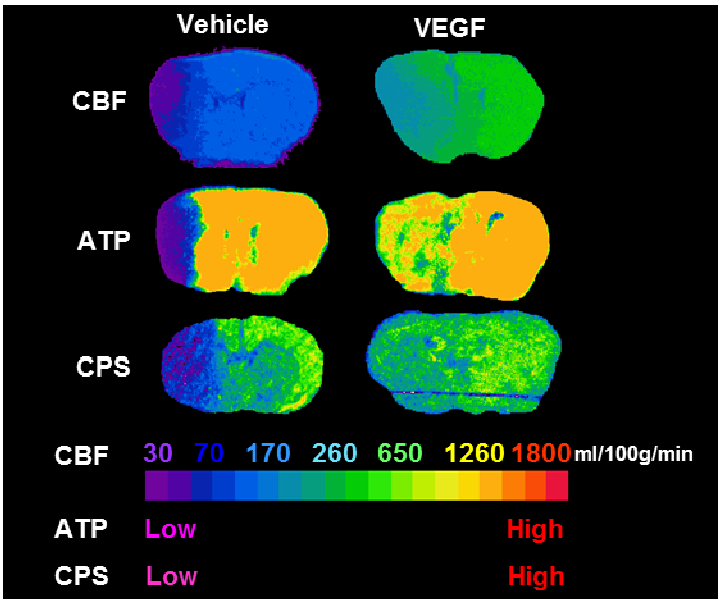


Figure 9. VEGF increases Rac1 activity in the ischemic brain. Rac1 G-LISA demonstrating increased Rac1 activity after 21 days of VEGF treatment, reflecting the maturation of functional vascular networks. Data are means ± SD (n=6-7 animals/ group). *p< 0.05/ **p< 0.01 compared with vehicle.

In view of the fact that VEGF treatment for 21 days promoted angiogenesis while simultaneously enhancing blood-brain barrier and cell matrix integrity, we further evaluated the effects of VEGF-induced angiogenesis on regional CBF and brain metabolism in mice prophylactically treated with VEGF for 21 days.

4.1.6 VEGF increases regional CBF in the ischemic cortex and striatum

The effects of VEGF-induced angiogenesis on brain hemodynamics were investigated by analysis of autoradiographic measurements of CBF. Increased regional CBF were noted in the ischemic cortex and striatum of VEGF compared with vehicle treated animals (Fig. 10 A), demonstrating that new vessel formation did indeed translate into a functional improvement of blood flow. Interestingly, increased CBF values were also observed in the contralateral non-ischemic striatum (Fig. 10 B), albeit capillary density was not increased by VEGF treatment suggesting that mechanisms other than capillary density contributed to changes in CBF.



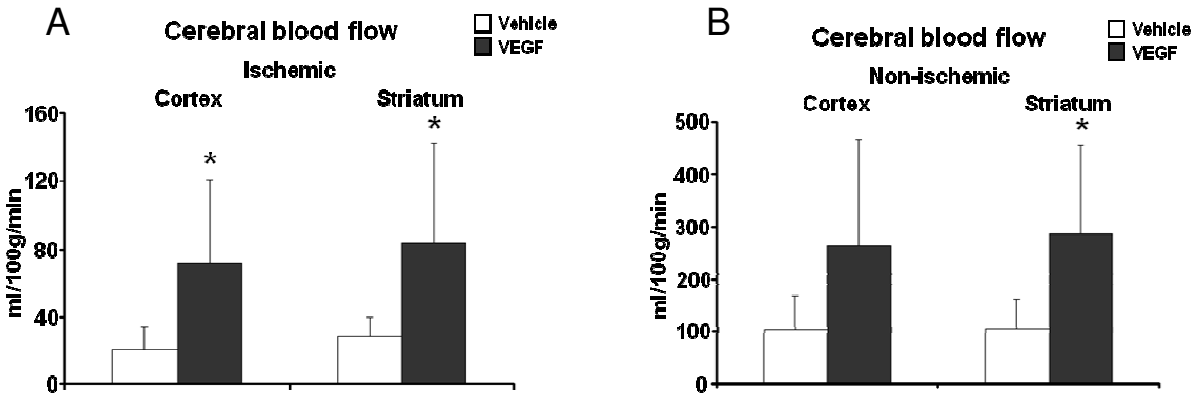


Figure 10 (for legend see page 53)

Figure 10. VEGF increases regional CBF after focal cerebral ischemia (**A**) CBF analysis in the ischemic cortex and striatum of mice subjected to 90 min MCA occlusion showing improvement of blood flow 21 days after initiation of VEGF treatment. (**B**) CBF analysis in the contralateral hemisphere revealing increased blood flow in the striatum but not in cortex after 21 days of VEGF delivery. Representative images are also shown (top). Data are means ± SD (n=6 animals/ group). *p< 0.05/ **p< 0.01 compared with vehicle.

4.1.7 VEGF-induced angiogenesis stabilizes the metabolic penumbra and prevents ATP depletion

To evaluate how the improvement of regional CBF influences regional cerebral energy states, CPS and ATP bioluminescence images were assessed. A stabilization of the metabolic penumbra, defined as brain tissue in which CPS is suppressed but ATP preserved (Fig. 11 A), as well as a reduction in the area of tissue exhibiting ATP depletion (Fig. 11 B) was found in VEGF but not in vehicle treated animals, thus indicating that blood flow changes resulted in a preservation of energy metabolism.

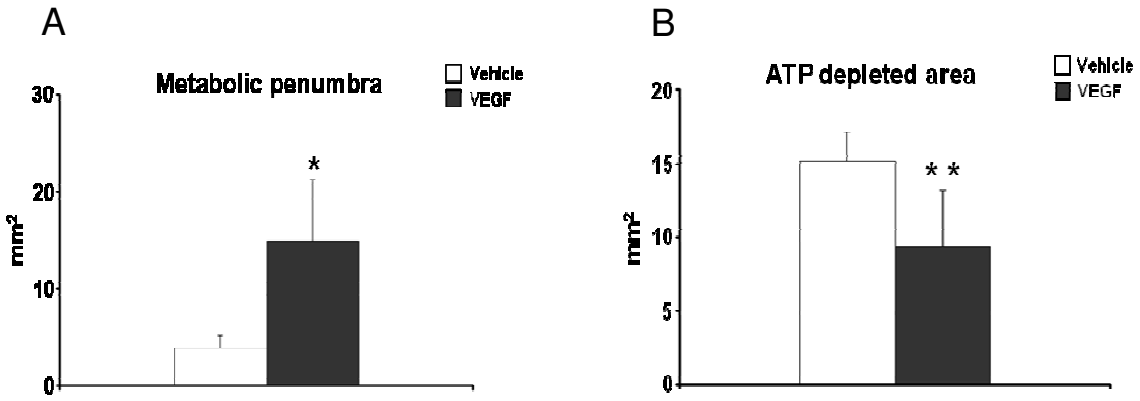


Figure 11. VEGF stabilizes the metabolic penumbra and prevents ATP depletion. (**A**) Preservation of the metabolic penumbra, defined as brain tissue in which CPS is suppressed but ATP preserved, due to VEGF-induced angiogenesis in animals after 21 days of VEGF delivery. (**B**) Reduction of ATP-depleted tissue in the same animals. Data are means ± SD (n=6 animals/ group). *p< 0.05/ **p< 0.01 compared with vehicle.

4.1.8 VEGF increases pericyte coverage of brain capillaries

To examine the functionality of cerebral blood vessels, pericyte coverage was analyzed. In the contralateral non-ischemic hemisphere, the vast majority of capillaries (~90%) were surrounded by pericytes (Fig. 12 B). In the ischemic tissue, this value was lower (~60%) in vehicle treated mice (Fig. 12 A). Interestingly, VEGF treatment did not affect the percentage of pericyte positive capillaries, when initiated 3 or 10 days prior to stroke, but markedly increased pericyte coverage of ischemic capillaries, when started 21 days before (Fig. 12 A), indicating that VEGF induces the formation of mature vessels. Two-way ANOVA revealed a significant effect of VEGF ($F_{1,30}$=8.981; $p< 0.01$) and a VEGF x treatment duration interaction effect for the percentage of pericyte covered vessels ($F_{2,30}$=3.709; $p< 0.05$).

In view of the ceiling effect of pericyte coverage in the contralateral non-ischemic tissue (Fig. 13B), where an increase in CBF, but not capillary density was found (see Figs. 5 and 10), a confocal data analysis was also performed, in which pericyte volumes determined in 3D stacks were related to the volumes of CD31+ cerebral capillaries. In this analysis, VEGF increased pericyte coverage in both hemispheres, when initiated 21 days before the stroke (Fig. 12 C, D). These data explained why VEGF increased CBF in the contralateral non-ischemic brain tissue although no increase in capillary density was seen. Two-way ANOVA revealed a VEGF x treatment duration interaction effect for the volume ratio ($F_{2,29}$=3.447; $p< 0.05$).

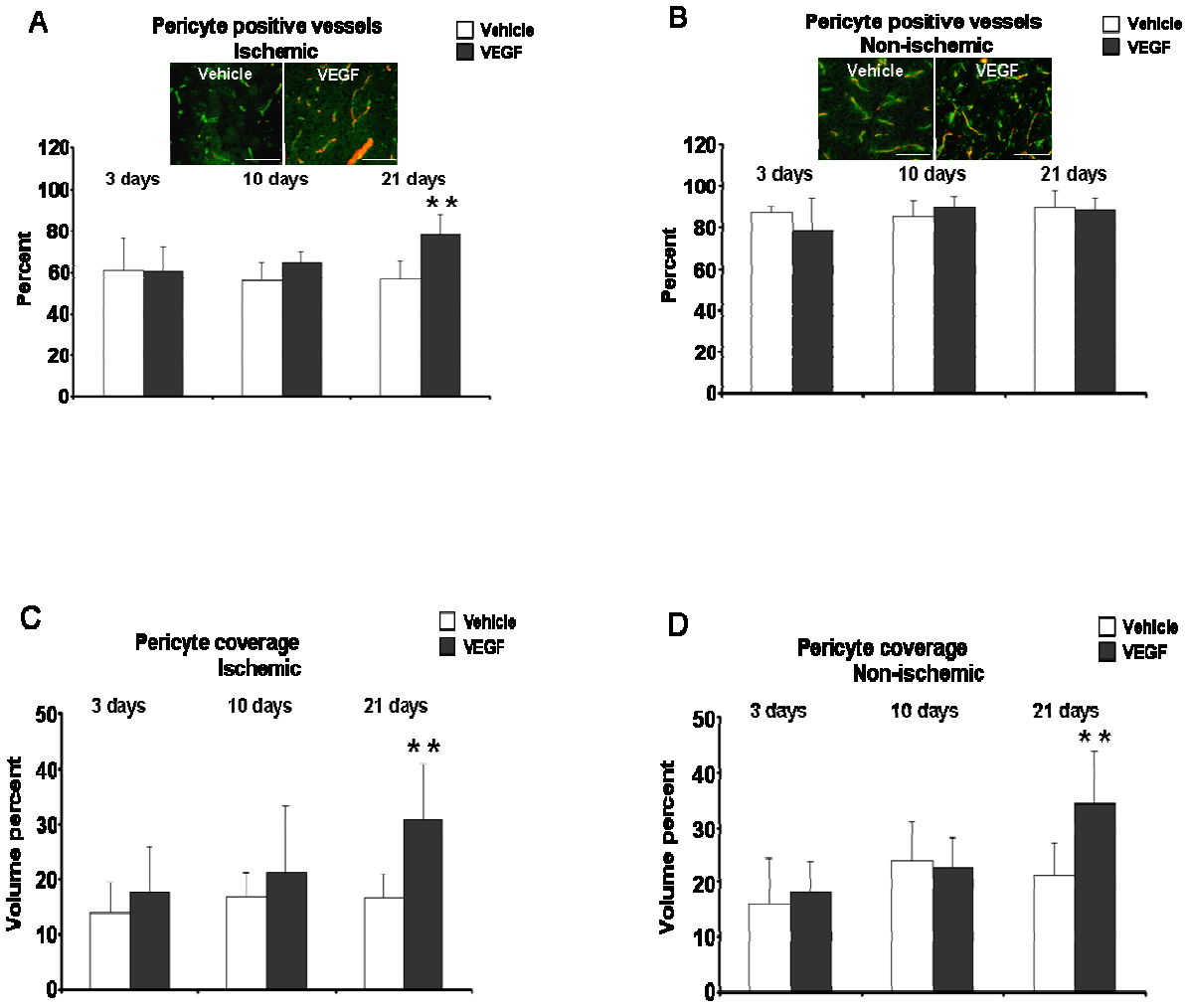


Figure 12. VEGF promotes pericyte coverage of brain endothelial cells. (**A**, **B**) Percentage of pericyte positive microvessels in the ischemic and contralesional non-ischemic brain tissue, (**C**, **D**) volume coverage of microvessels by pericytes, Representative microphotographs from animals treated with vehicle or VEGF for 21 days are also shown (CD31 in **A** and **B** in green/ desmin in **A** and **B** in red). Data are means ± SD (n=6-7 animals/ group). *p< 0.05/ **p< 0.01 compared with vehicle. Bar, 100 µm.

4.1.9 VEGF increases the junctional protein N-cadherin on cerebral microvessels

The alignment of pericytes to endothelial cells is mediated by the junctional protein N-cadherin (Winkler et al., 2011). To evaluate whether N-cadherin was influenced by VEGF, Western blots were prepared with capillary extracts which revealed increased N-cadherin expression in microvessels that had been harvested 21 days after the initiation of VEGF treatment (Fig.13).

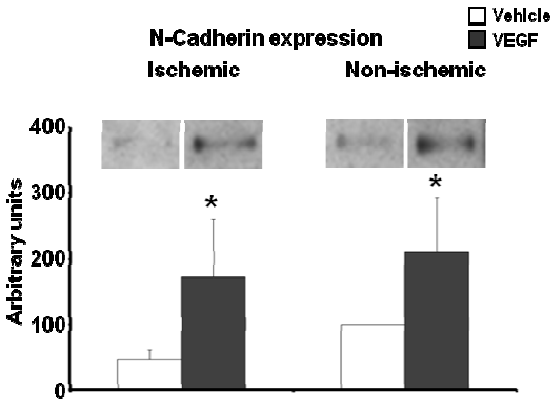


Figure 13. VEGF increases the expression of the junctional protein N-cadherin on cerebral microvessels. N-cadherin expression on cerebral microvessels in animals treated with VEGF for 21 days. Representative blots from animals treated with vehicle or VEGF for 21 days are also shown. Data are means ± SD (n=4 independent blots). *p< 0.05/ **p< 0.01 compared with vehicle.

4.1.10 VEGF does not influence apoptotic death of pericytes

To rule out whether changes in pericyte coverage in the ischemic tissue were partly due to reduced pericyte death, numbers of desmin/ TUNEL and desmin/ cleaved caspase-3 double positive pericytes were analyzed. Notably, VEGF treatment neither influenced deoxyribonucleic acid (DNA) fragmentation nor caspase-3 activation (Fig. 14).

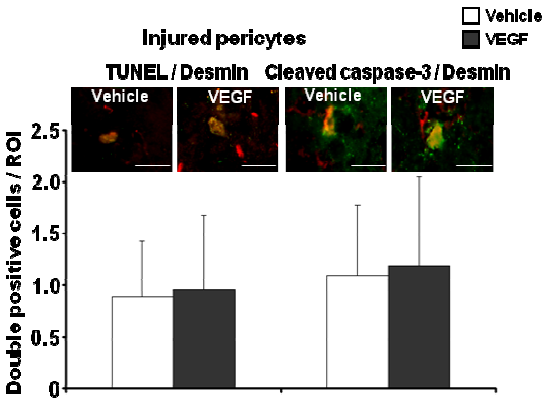


Figure 14. VEGF does not influence pericytic injury. DNA-fragmented (i.e., TUNEL+) and apoptotic (i.e., cleaved caspase-3+) pericytes, evaluated using the pericyte marker desmin in animals treated with VEGF for 21 days. Note the absence of effects of VEGF on pericyte injury. Representative microphotographs from animals treated with vehicle or VEGF for 21 days are also shown (TUNEL and cleaved caspase-3 in green/ desmin in red). Data are means ± SD (n=6-7 animals/ group). *p< 0.05/ **p< 0.01 compared with vehicle. Bar, 40 µm.

4.1.11 VEGFR2 and VEGFR1 are not expressed on mouse brain pericytes

Since VEGF has previously been shown to induce pericyte ablation in Matrigel assays, which was interpreted as consequence of VEGFR2/ PDGFRβ interaction on pericytes (Greenberg et al., 2008), the question arose whether pericytes in the C57BL6/j mouse strain we used expressed VEGFR2 or VEGFR1. In immunostainings, we were unable to detect VEGFR2 and VEGFR1 on pericytes (Fig. 15 A, B).

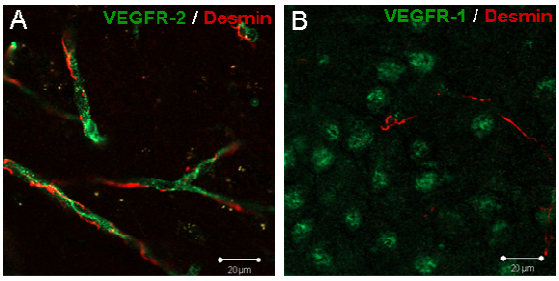


Figure 15. Absence of VEGFR2 and VEGFR1 on pericytes. Immunofluorescence photographs revealing VEGFR2 (**A**) and VEGFR1 (**B**) (both in green) on ischemic endothelial cells (in case of VEGFR2 in **A**) or parenchymal cells (in case of VEGFR1 in **B**), but not on pericytes (in red). Bar, 20 µm.

4.2 Effect of hyperlipidemia on VEGF-induced angiogenesis

4.2.1 VEGF-induced capillary formation is blunted by hyperlipidemia

To examine how the i.c.v. delivery of VEGF influences microvascular networks in wildtype and ApoE$^{-/-}$ mice, brain capillary density was analysed by CD31 immunohistochemistry. VEGF increased capillary density in wildtype mice on normal diet and Western diet and ApoE$^{-/-}$ mice on normal diet, but not in ApoE$^{-/-}$ mice on Western diet (Fig.16 A,B). Three-way ANOVA showed a significant interaction effect for VEGF x ApoE x dietary status on capillary density ($F_{1,39}$=4.444; $p<0.05$), demonstrating that angiogenesis was prevented by hyperlipidemia, most strongly in animals exhibiting the highest cholesterol levels (i.e., ApoE$^{-/-}$ mice on Western diet).

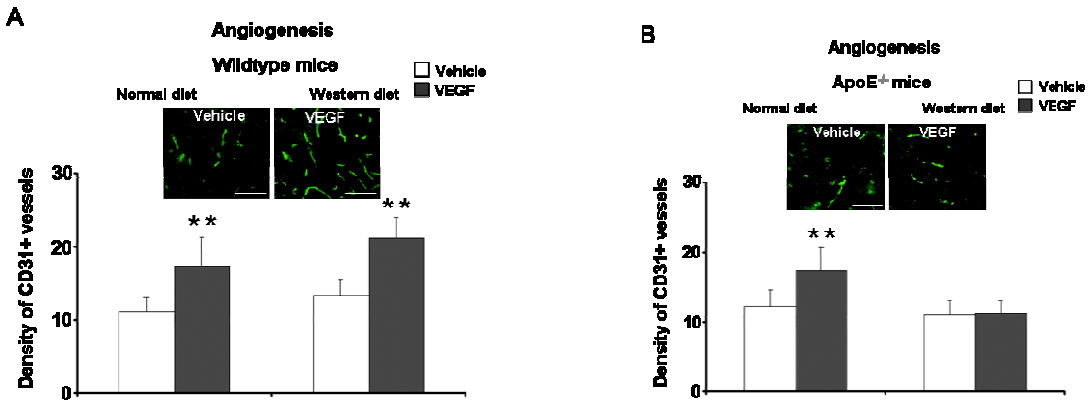


Figure 16. Hyperlipidemia prevents VEGF-induced brain capillary formation. (**A**, **B**) CD31 immunohistochemisty showing an increased capillary density in VEGF treated wildtype mice kept on normal and Western diet and ApoE$^{-/-}$ mice on normal diet. Capillary formation is blunted in ApoE$^{-/-}$ mice on Western diet that exhibit the highest plasma cholesterol levels between all groups. Representative microphotographs from wildtype mice kept on normal diet and ApoE$^{-/-}$ mice on Western diet are also shown. Data are means±SD (n=6-7 animals/ group). *p<0.05/ **p<0.01 compared with corresponding vehicle. Bar, 100 µm.

4.2.2 Hyperlipidemia induces post-ischemic hypoperfusion

To evaluate the effects of hyperlipidemia on histopathological brain injury and blood-brain barrier integrity, mice treated with vehicle or VEGF were exposed to 90 min MCA occlusion followed by 24 hours reperfusion. LDF recordings taken during and after MCA occlusion revealed reproducible ischemic impacts that did not differ between vehicle and VEGF treated mice (Fig.17 A,B). Post-ischemic LDF values were lower in animals on Western diet than animals on normal diet (Fig.17 A,B).

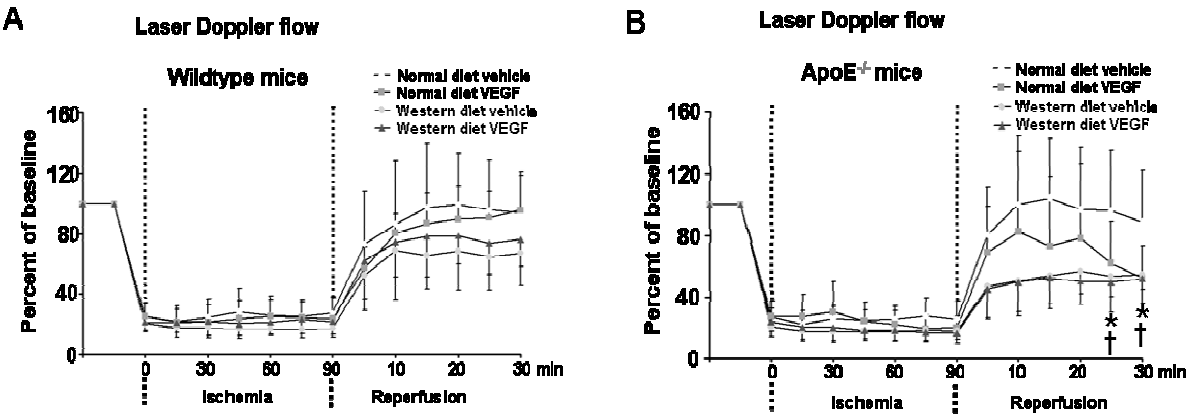


Figure 17. Hyperlipidemia induces post-ischemic hypoperfusion. LDF recordings confirming the reproducibility of MCA occlusions. Note that LDF values after reperfusion are lower in animals kept on Western diet than in animals kept on normal diet. Data are means ± SD (n=6-7 animals/ group). *$p<0.05$ for VEGF treated normal diet compared with vehicle treated normal diet; †$p<0.05$ for vehicle treated Western diet compared with vehicle treated normal diet.

4.2.2 Hyperlipidemia abolishes VEGF-induced neuroprotection

Infarct measurements on cresyl violet stainings at 24 hours after reperfusion revealed that VEGF reduced infarct volume in wildtype mice kept on normal diet (Fig.19A), which is in line with earlier results of our group (Wang et al., 2005; Zechariah et al., 2011). Conversely, VEGF did not influence infarct size in wildtype mice on Western diet, ApoE$^{-/-}$ mice on normal diet or ApoE$^{-/-}$ mice on Western diet (Fig.18 A,B).

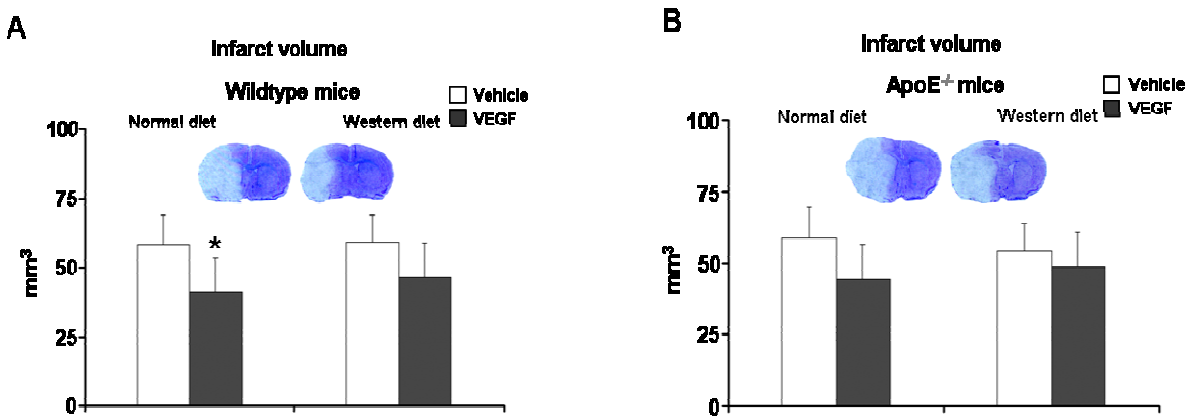


Figure 18. Hyperlipidemia abolishes VEGF-induced neuroprotection. (**A**, **B**) Infarct volume of mice submitted to 90 minutes MCA occlusion followed by 24 hours reperfusion showing VEGF-induced reduction of ischemic injury in wildtype mice on normal diet, but not in wildtype mice on Western diet and in ApoE$^{-/-}$ mice on normal or Western diet that exhibit hyperlipidemia. Representative images from wildtype mice kept on normal diet and ApoE$^{-/-}$ mice on Western diet are also shown. Data are means ± SD (n=6-7 animals/ group). *p<0.05/ **p<0.01 compared with corresponding vehicle.

4.2.3 Hyperlipidemia abolishes VEGF-induced preservation of blood-brain barrier integrity

Serum IgG extravasation evaluated using immunohistochemistry revealed the status of BBB under conditions of hypercholestremia. The attenuation of brain injury in wildtype mice on normal diet was also associated with reduced IgG extravasation. The preservation of BBB induced by VEGF was abolished under conditions of hyperlipidemia leading to increased extravasation of serum proteins (Fig.19 A,B).

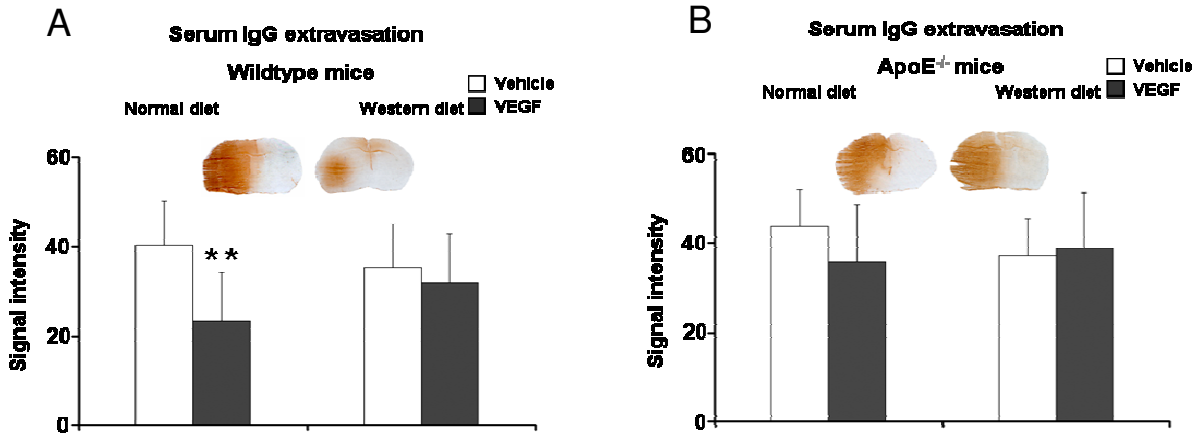


Figure 19. Hyperlipidemia abolishes VEGF-induced blood-brain barrier preservation. (**A**, **B**) Immunohistochemistry aganist serum IgG, demonstrating decreased serum IgG extravasation by VEGF in wildtype mice on normal diet, but not in wildtype mice on Western diet and ApoE$^{-/-}$ mice on normal or Western diet. In these animals, tissue samples derived from the ischemic MCA territory (striatum and overlying cortex) were analysed. Representative images from wildtype mice kept on normal diet and ApoE$^{-/-}$ mice on Western diet are also shown. Data are means ± SD (n=6-7 animals/ group). *p<0.05/ **p<0.01 compared with corresponding vehicle.

4.2.4 Hyperlipidemia prevents VEGF-induced extracellular matrix preservation

To assess how hyperlipidemia influences extracellular matrix integrity after VEGF treatment, MMP-9 activity was analysed by gelatin zymography. In line with a previous study (Reitmeier et al., 2012), VEGF reduced MMP-9 activity both in ischemic tissue samples obtained from wildtype and ApoE$^{-/-}$ mice, yet more strongly in animals kept on normal diet than on Western diet (Fig.20 A,B), indicating that hyperlipidemia promoted extracellular matrix breakdown. Indeed, VEGF treated ApoE$^{-/-}$ mice on Western diet exhibited significantly higher MMP-9 activity than ApoE$^{-/-}$ mice on normal diet (Fig.20 B).

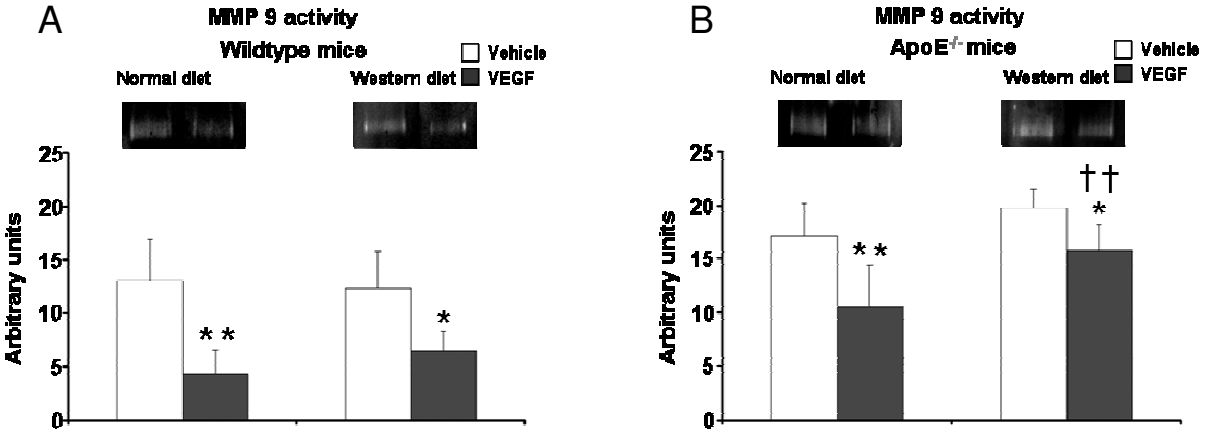
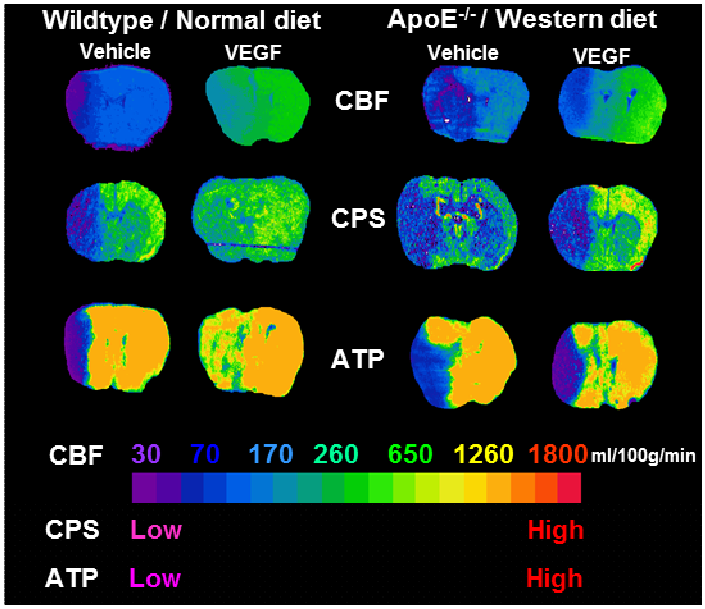


Figure 20. Hyperlipidemia abolishes VEGF-induced extracellular matrix preservation. (**A**, **B**) Gelatin zymography exhibiting deactivation of MMP-9 by VEGF in wildtype mice on normal diet that is attenuated in wildtype mice on Western diet and ApoE$^{-/-}$ mice on normal or Western diet. Tissue samples derived from the ischemic MCA territory (striatum and overlying cortex) were analysed. Representative images from wildtype mice kept on normal diet and ApoE$^{-/-}$ mice on Western diet are also shown. Data are means ± SD (n=6-7 animals/ group). *p<0.05/ **p<0.01 compared with corresponding vehicle. ††p<0.01 compared with corresponding normal diet.

4.2.5 Hyperlipidemia attenuates VEGF-induced improvement of regional CBF

To elucidate the effect of VEGF-induced angiogenesis on brain hemodynamics during a subsequent stroke, CBF autoradiograms were analysed at 60 min after MCA occlusion. Increased regional CBF was noted in the MCA territory of VEGF treated wildtype mice on normal diet but not in VEGF treated ApoE$^{-/-}$ mice on Western diet (Fig.21 A), indicating a lack of functional hemodynamic improvements in animals with hyperlipidemia. Two-way ANOVA demonstrated a significant effect of VEGF ($F_{1,20}$=8,705; p<0.01), but no interaction effect with ApoE/ dietary status on regional CBF.



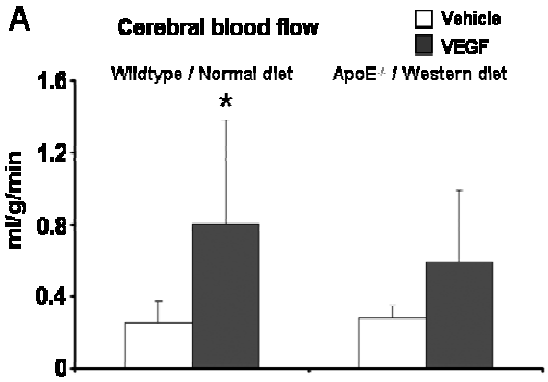


Figure 21 (for legend see page 65)

Figure 21. Hyperlipidemia abolishes VEGF-induced improvement of cerebral blood flow. (**A**) Regional CBF autoradiography showing increased blood flow values in ischemic brain tissue of VEGF treated wildtype mice on normal diet that are attenuated in ApoE$^{-/-}$ mice on Western diet that exhibit hyperlipidemia. Animals were exposed to 90 minutes MCA occlusion, followed by 60 minutes reperfusion. Tissue samples derived from the ischemic MCA territory (striatum and overlying cortex) were evaluated. Representative autoradiographies and bioluminescence images are also shown. Data are means ± SD (n=6 animals/ group). *p<0.05/ **p<0.01 compared with corresponding vehicle.

4.2.6 Hyperlipidemia abolishes VEGF-induced stabilization of metabolic penumbra

To evaluate how the improvement of regional CBF influences regional cerebral energy state, CPS and ATP bioluminescence images were assessed. A stabilization of the metabolic penumbra, defined as brain tissue in which CPS was suppressed but ATP preserved, was noticed in VEGF treated wildtype mice on normal diet but not in ApoE$^{-/-}$ mice on Western diet (Fig.22 A). Two-way ANOVA showed a significant VEGF x ApoE/ dietary status interaction effect ($F_{1,14}$=4.663; p<0.05), indicating that hyperlipidemia promotes secondary brain infarction. Indeed, the tissue area exhibiting ATP depletion after 60 minutes of reperfusion was considerably smaller in normolipidemic wildtype than hyperlipidemic ApoE$^{-/-}$ mice (Fig.22 B), although this difference still failed to show significance at this time point.

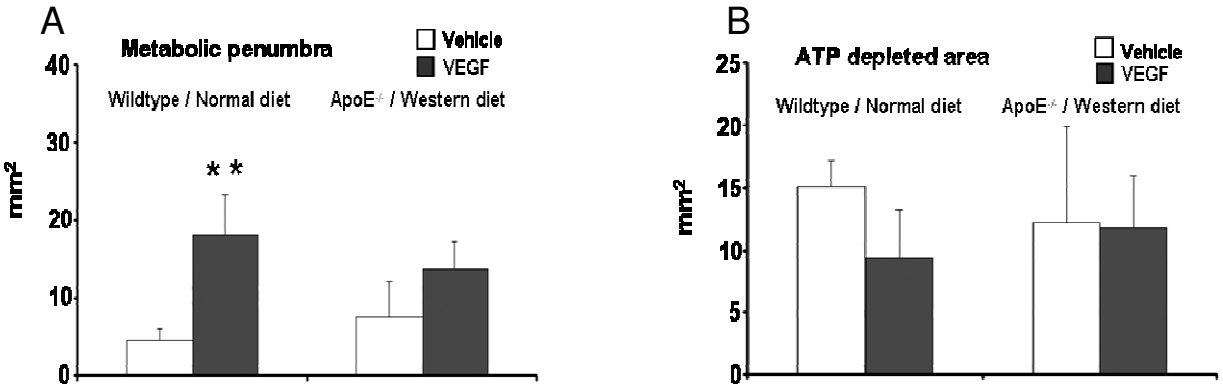


Figure 22 (for legend see page 66)

Figure 22. Hyperlipidemia abolishes VEGF-induced preservation of brain energy state. (**A**) Metabolic penumbra, defined as tissue area in which cerebral protein synthesis is suppressed, but ATP preserved, exhibiting the viability of the metabolic penumbra as a result of the enhanced blood flow in VEGF treated wildtype mice on normal diet, but not in hyperlipidemic ApoE$^{-/-}$ mice on Western diet. (**B**) Tissue area exhibiting ATP depletion, revealing reduced breakdown of the cerebral energy state in VEGF treated normolipidemic wildtype mice, but not in hyperlipidemic ApoE$^{-/-}$ mice. Animals were exposed to 90 minutes MCA occlusion, followed by 60 minutes reperfusion. Tissue samples derived from the ischemic MCA territory (striatum and overlying cortex) were evaluated. Data are means ± SD (n=6 animals/ group). *p<0.05/ **p<0.01 compared with corresponding vehicle.

4.2.7 Hyperlipidemia prevents VEGF-induced pericyte alignment on brain capillaries

To examine the functionality of cerebral blood vessels in addition to structural growth, pericyte coverage was analysed in brain tissue samples submitted to focal cerebral ischemia. In the ischemic tissue, the percentage of cerebral microvessels surrounded by pericytes was around 60% in 20 µm thick cryostat sections (Fig.24 A,B). Interestingly, VEGF treatment increased the percentage of pericyte+ capillaries in wildtype mice on normal diet (relatively by ~30%), but not in wildtype mice on Western diet or ApoE$^{-/-}$ mice on normal or Western diet (Fig.23 A,B). Three-way ANOVA revealed a significant VEGF x ApoE x dietary status interaction effect ($F_{1,41}$=4.487; p<0.05).

To further corroborate these findings, we subsequently performed a confocal data analysis, in which pericyte volumes determined in 3D stacks were related to volumes of CD31^{+} cerebral capillaries. This study again revealed an increased pericyte coverage of brain endothelial cells in VEGF treated wildtype mice on normal diet, but not in wildtype mice on Western diet or any of the ApoE$^{-/-}$ mice (Fig.23 C,D). Three-way ANOVA again confirmed a VEGF x ApoE x dietary status interaction effect ($F_{1,41}$=7,325; p<0.05), confirming that hyperlipidemia disturbed the alignment of pericytes with endothelial cells.

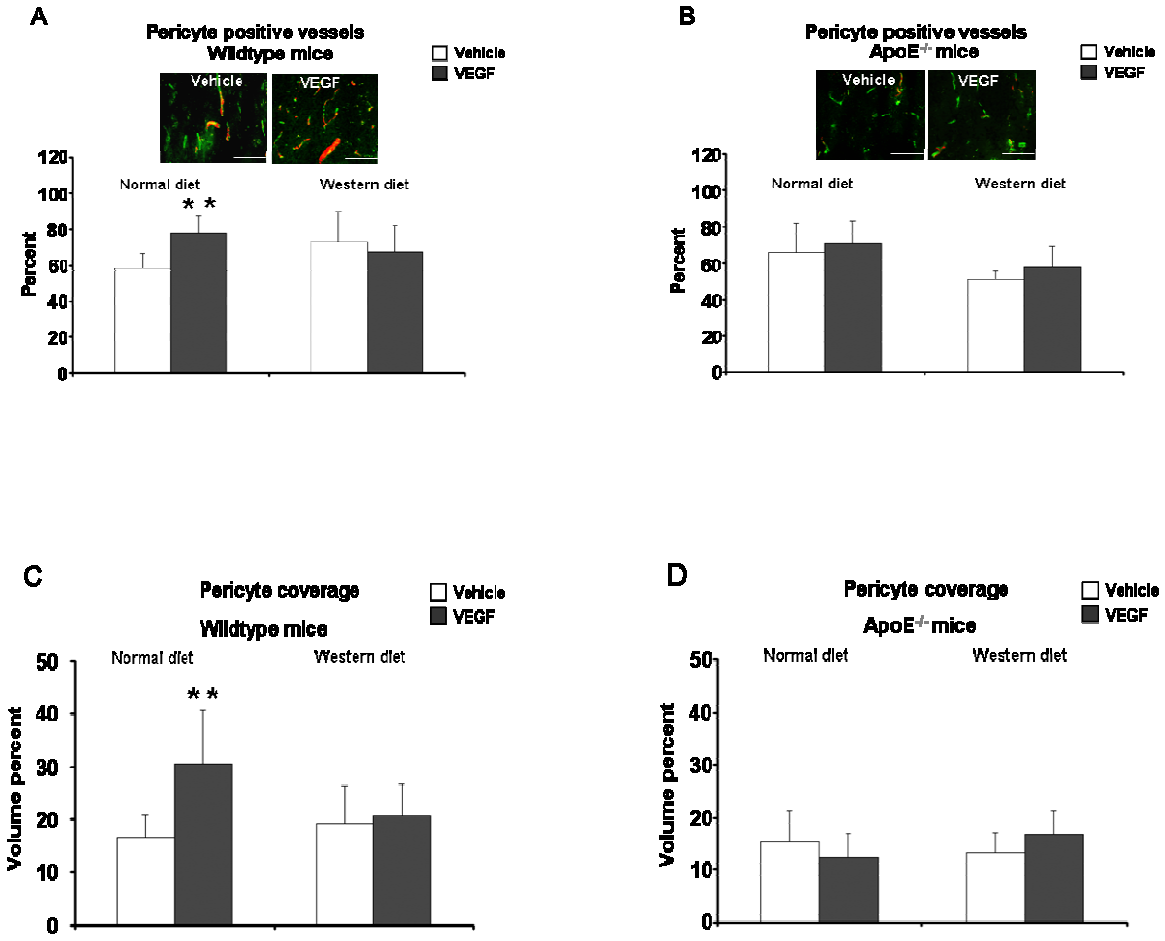


Figure 23. Hyperlipidemia prevents VEGF-induced pericyte alignment on brain capillaries. (**A**, **B**) Percentage of cerebral microvessels surrounded by pericytes in 20 µm cryostat sections, (**C**, **D**) volume ratio of pericyte coverage of ischemic brain capillaries determined in 3D stacks. Note the increased pericyte coverage in VEGF treated wildtype mice on normal diet, but not in wildtype mice on Western diet or in ApoE$^{-/-}$ mice on normal or Western diet that exhibit hyperlipidemia (**A-D**). Animals were submitted to 90 minutes MCA occlusion followed by 24 hours reperfusion. Tissue samples derived from the ischemic MCA territory (striatum and overlying cortex) were evaluated. Representative microphotographs are also shown (CD31 in green/ desmin in red). Data are means±SD (n=6-7 animals/ group). *p<0.05/ **p<0.01 compared with vehicle. Bar, 100 µm.

4.2.8 Hyperlipidemia attenuates N-cadherin expression in cerebral microvessels

The physical interaction of pericytes with endothelial cells is mediated by the junctional protein N-cadherin (Winkler et al., 2011). To evaluate whether N-cadherin expression was altered by an increase in blood lipids, Western blots were prepared with capillary extracts obtained from normolipidemic and hyperlipidemic wildtype and ApoE$^{-/-}$ mice. This study revealed that VEGF potently increased N-cadherin levels in ischemic microvessels obtained from wildtype mice on normal diet, but not in any of the hyperlipidemic groups (Fig.24 A,B). The lack of N-cadherin expression provides a mechanism for the disturbed pericyte coverage of endothelial cells in hyperlipidemia.

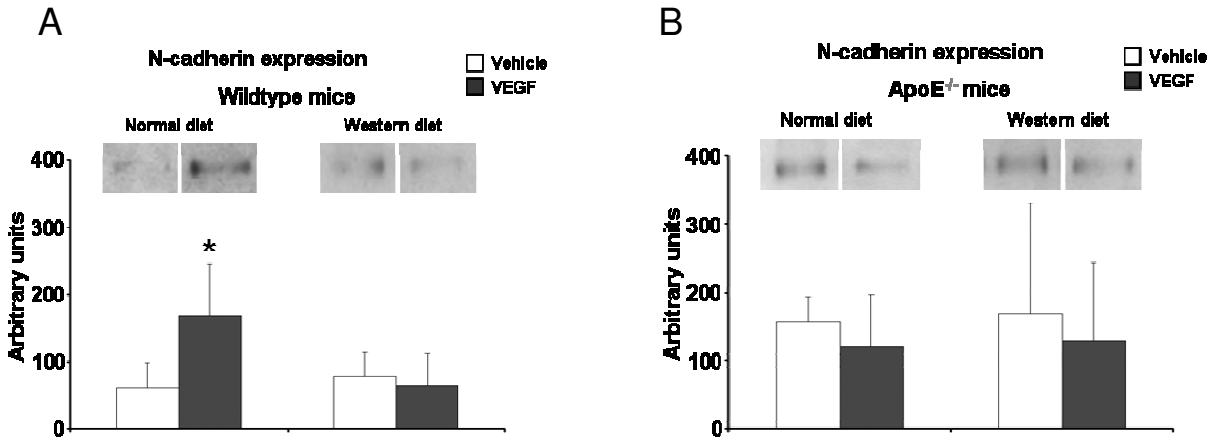


Figure 24. Hyperlipidemia decreses N-cadherin expression affecting pericyte alignment on brain capillaries. (**A**, **B**) N-cadherin expression in ischemic cerebral microvessels of wildtype and ApoE$^{-/-}$ mice on normal or Western diet. Also note the increased expression of N-cadherin, which physically links the pericytes with endothelial cells, in VEGF treated wildtype mice on normal diet, but not in hyperlipidemic wildtype mice on Western diet or ApoE$^{-/-}$ mice. Animals were submitted to 90 minutes MCA occlusion followed by 24 hours reperfusion. Microvessels derived from the ischemic MCA territory (striatum and overlying cortex) were evaluated. Representative blots are also shown. Data are means±SD (n=4 independently processed blots). *p<0.05/ **p<0.01 compared with vehicle.

5 Discussion

Understanding the pathophysiological mechanisms involved in the innate recovery process of the brain is crucial for developing therapeutic strategies. Angiogenesis is a crucial component of the neurovascular remodeling occurring after cerebral ischemia. Assessing the role of major risk factors such as atherosclerosis on remodeling promoting strategies is decisive for minimising translational failures.

By using a multiparametric approach in wildtype animals we show that VEGF induces the formation of new vessels leading to increase in cerebral perfusion and preservation of the cerebral energy state after focal cerebral ischemia. Our results also demonstrated increased pericyte coverage of cerebral capillaries after VEGF treatment by augmenting N-cadherin expression. Our observations demonstrated the ability of VEGF to induce neurovascular remodelling; even though the application strategies and compromising effects of risk factors still needs to be optimised and investigated.

By utilising hyperlipidemic wildtype and $ApoE^{-/-}$ animals we furthermore demonstrate that VEGF induced new vessel formation is compromised under conditions of hyperlipidemia. Dysfunctional angiogenesis was followed by decreased cerebral perfusion and less preservation of the cerebral energy state. Additionally, hyperlipidemia affected pericyte coverage of brain vessels by decreasing N-cadherin expression. Our observations clearly demonstrate the shortcomings of translational studies in ischemic stroke which utilise normolipidemic, healthy animals which do not reflect the pathophysiology encountered in clinical situations.

5.1 VEGF promotes pericyte coverage of brain capillaries, improves cerebral blood flow during subsequent focal cerebral ischemia, and preserves the metabolic penumbra

By applying prophylactic VEGF for up to 21 days, we have shown that VEGF induces the formation of mature, functional blood vessels, which enables the brain to better cope with subsequent ischemic episodes, thus enhancing regional CBF, stabilizing cerebral energy state and preventing brain infarction. In our study, increased brain capillary densities were noted within 10 days of initiation of VEGF treatment, which were accompanied by enhanced pericyte coverage of endothelial cells within 21 days. The induction of angiogenesis closely paralleled tissue survival, providing evidence of links between new vessel formation, hemodynamic changes and tissue preservation.

Previous investigations have demonstrated the ability of VEGF to stimulate the production of capillaries in intact brain (Zhao et al., 2010; Yano et al., 2005; Wang et al., 2005) and after focal cerebral ischemia (Reitmeir et al., 2012; Wang et al., 2007c; Sun et al., 2003). In our study VEGF delivery increased the number of new born vessels after 10 and 21 days but not after 3 days of treatment which is line with previous reports revealing that increase of new vessels in the brain starts only around 48 hours after stroke (Seylaz et al., 1999; Marti et al., 2000; Pinard et al., 2000). Additionally, by utilising autoradiographic techniques we now were able to show that selective induction of vessels by chronic treatment with VEGF leading to the increase in CBF and to the preservation of the cerebral energy state after focal cerebral ischemia.

Our observation that induced angiogenesis increases blood flow in the ischemic brain contrasts with earlier findings of our own group using a transgenic mouse line expressing human VEGF under control of a neuron-specific NSE promoter (Wang et al., 2005). In that mouse line, increased regional CBF, evaluated using 4-iodo-N-methyl-[14C] antipyrine, was described only in non-ischemic brain areas outside the MCA territory, while regional CBF within the ischemic MCA territory was actually reduced (Wang et al., 2005). These data were interpreted as an indication of a hemodynamic steal flow phenomenon induced by enhanced

angiogenesis (Wang et al., 2005). Indeed, human VEGF-transgenic mice exhibited an increased brain capillary density throughout the brain, which might explain why blood flow was redirected into non-ischemic areas. While capillary density was increased by up to 100% or even more in human VEGF-transgenic mice, only mildly increased blood flow values were noted in this mouse line under physiological conditions during hypercapnia (Vogel et al., 2004). These findings raised doubts whether microvascular networks are adopted to tissue needs in this mouse line. In a magnetic resonance imaging study, acute VEGF infusion was previously shown to induce transient CBF increases in ischemic brain tissue lasting up to 3 hours (Zhang et al., 2000), which however was a consequence of VEGF-induced vasorelaxation rather than of induced vascular growth.

The capability of blood vessels to respond to tissue needs depends on proper interactions between endothelial cells with pericytes (Kaya et al., 2005), which, as we show, are enhanced by VEGF treatment. The increased pericyte coverage of brain capillaries was associated with an enhanced expression of the junctional protein N-cadherin. N-cadherin promotes the alignment of pericytes to endothelial cells by enabling direct physical interactions between both cell types (Kaya et al., 2005). Our observation of increased pericyte coverage indicates that therapeutic angiogenesis may have beneficial effects beyond elevating capillary density. In our study we observed an enhanced pericyte coverage following VEGF treatment also in areas not exhibiting vascular sprouting. Notably in the contralateral striatum, at distance to the VEGF infusion, potently increased CBF values were noticed despite lack of angiogenesis, which is well explained by the recruitment of pericytes. Our results are in contrast to observations following VEGF treatment in a model of PDGF-BB-induced angiogenesis, where a loss of pericyte coverage was reported, which was mediated by the deactivation of PDGFRβ by VEGFR2 (Greenberg et al., 2008). In the present study, we have not been able to detect VEGFR2 on brain pericytes, neither under physiological conditions nor following stroke. Differences of the growth factors used –combined VEGF/PDGF-BB delivery vs. VEGF delivery only– together with differences in the experimental systems – Matrigel assay vs. focal cerebral ischemia– may explain diverging results.

In addition to increased new vessel formation and perfusion, chronic VEGF delivery for 10 and 21 days also reduced tissue injury after focal cerebral ischemia. Our results are in line with previous observations where reductions in infarct volume (Li et al., 2009; Wang et al., 2007c; Shen et al., 2006; Kaya et al 2005; Sun et al., 2003; Hayashi et al., 1998; Emerich et al., 2010), reduced secondary neuronal degeneration (Herz et al., 2012), DNA fragmented cells (Shen et al., 2006), TUNEL positive cells (Kaya et al., 2005) and reduction in cell shrinkage and DNA cleavage (Sun et al., 2003) were observed after local delivery of VEGF. The ability of VEGF to influence tissue injury could be explained by the presence of functional newborn vessels facilitating increased perfusion of tissue and the reduction of infarct volume after focal cerebral ischemia.

A major concern regarding VEGF-induced angiogenesis is the increased BBB permeability that is more pronounced following acute (Zhang et al., 2000; Kaya et al., 2005) than prophylactic (Wang et al., 2005; Bellomo et al., 2003) or post-acute (Zhang et al., 2000) VEGF delivery, and more pronounced after intravenous (Zhang et al., 2000; Kaya et al., 2005) than local brain (Wang et al., 2005; Bellomo et al., 2003) administration. VEGF pre-treatment for 6-12 days have been shown to decrease brain edema in gerbils (Bellomo et al., 2003) while increased BBB damage was observed when delivered during the acute phase after stroke (Zhang et al., 2000) in rats. Furthermore, VEGF antagonisation during the acute phase of stroke resulted in decreased edema formation (van Bruggen et al., 1999). Additionally, local delivery of VEGF, 1-3 days after stroke, was observed to reduce infarct size, improve neurological performance, enhance delayed survival of newborn neurons and to stimulated angiogenesis after focal cerebral ischemia in rats (Sun et al., 2003). In our study, the extravasation of serum IgG was markedly reduced in animals with 21 days of VEGF treatment but not after 3 or 10 days. Even though the number of new born vessels was increased after 10 days of treatment, pericyte coverage was only increased after 21 days of VEGF treatment which explains the reduction in IgG extravasation in these animals owing to better preservation of BBB by enhanced pericyte coverage. These data reveal that the duration of VEGF exposure critically influences the integrity of newly formed vessels.

Using MMP-9 gelatin zymography and protein expression analysis we show that long term VEGF treatment diminishes MMP-9 activity and transiently reduces inflammation after focal ischemia. MMP-9 activation is often linked to changes in the BBB by disrupting the cell matrix interactions leading to brain swelling and intracerebral haemorrhage (Wang et al., 2003; Cheng et al., 2006; Burggraf et al., 2007). Selective MMP inhibition leads to decreased brain injury, brain swelling, BBB breakdown and better functional outcome after focal cerebral ischemia in rats (Nagel et al., 2011). Specific MMP-9 inhibition alleviated BBB disruption, decreased brain edema and inhibited the production of inflammatory cytokines after cardiopulmonary resuscitation in rats (He et al., 2009). A recent investigation have demonstrated that post-acute delivery of VEGF attenuated CD45+ leukocyte infiltrates and diminished the microglial activation by downregulation of a board set of inflammatory cytokines and chemokines (Herz et al., 2012). The action of VEGF on BBB breakdown has been shown to depend on the route and time point of delivery (Hermann and Zechariah, 2009). According to our study, prophylactic local delivery of VEGF leads to preservation of BBB integrity and to the decrease in inflammation after focal cerebral ischemia.

Long term VEGF treatment increased Rac1 activity in our study, which is known to mediate endothelial cell motility, proliferation, survival, and endothelial barrier formation. Endothelial-specific Rac1 insufficient mice show impaired angiogenesis (Johanna et al., 2010). Rac1 activity is also involved in the degenerative process and long term recovery after cerebral ischemia (Sawada et al., 2008). An inactive state of Rac1 was correlated with decreased survival signalling, significant tauopathy along with learning and memory alterations after global cerebral ischemia in rats; an effect which was reversed after normalization of Rac1 levels (Sawada et al., 2008). In our study, increase in Rac1 activity correlated with increase in new vessel formation and preservation of the BBB integrity, indicating that the survival promoting effects of VEGF, at least partially, was mediated through the Rac1 GTPase pathway. As such, Rac1 GTPase pathway could be a specific target for future therapeutic interventions.

As we have evaluated therapeutic angiogenesis in hitherto healthy adolescent mice with intact vascular networks, the question remained whether the data obtained could be translated to the situation in mice with pre-existing atherosclerosis. Hence, in order to understand the effect of hyperlipidemia on therapeutic angiogenesis, we chose to compare normolipidemic wildtype animals treated with VEGF for 21 days (the group which showed robust increase in CBF, pericyte coverage and BBB integrity in the first part of our study) with hyperlipidemic wildtype and ApoE$^{-/-}$ animals.

5.2 Hyperlipidemia attenuates VEGF-induced angiogenesis, impairs cerebral blood flow and disturbs stroke recovery via decreased pericyte coverage of brain endothelial cells

Using a multiparametric approach combining histochemical, autoradiographic, bioluminescence and molecular biological studies we herein show that VEGF-induced angiogenesis is compromised by hyperlipidemia, translating into the loss of hemodynamic improvements following subsequent stroke injuries, the breakdown of the metabolic penumbra and brain infarction. Furthermore, N-cadherin expression was reduced on cerebral microvascular cells exposed to hyperlipidemia that was associated with decreased coverage of endothelial cells with pericytes, demonstrating that blood vessels were dysfunctional.

As ischemic stroke results from the gradual accumulation of many factors, the observed benefits of therapeutic angiogenesis in healthy animals could not be directly translated to the clinical setting. In order to address this issue, we now utilised wildtype and ApoE$^{-/-}$ mice fed with regular or cholesterol-rich chow to induce different degrees of hyperlipidemia. Our data might provide an explanation for the poor effects of angiogenic therapies in human patients with symptomatic atherosclerosis (Simons and Ware, 2003; Hermann and Zechariah, 2009; Potente et al., 2011). These patients frequently suffer from hyperlipidemia and particularly brain vessels are affected by atherosclerotic plaques (Turan et al., 2010; Park et al., 2011).

In our study, the ability of VEGF to induce new vessel formation was blunted under conditions of severe hyperlipidemia. Interestingly, new vessel formation was preserved in the mildly hyperlipidemic animals. Impaired spontaneous angiogenesis has previously been noted under conditions of hyperlipidemia (van Belle et al., 1997; Duan et al., 2000; Jang et al., 2000). Thus, reduced vessel densities have been reported in hyperlipidemic rats exposed to hindlimb ischemia, which was attributed to reduced nitric oxide activity in hyperlipidemic ischemic tissue (Duan et al., 2000). Consequences of hyperlipidemia on brain angiogenesis and on the responses of blood vessels to VEGF were so far unknown.

In mice expressing human VEGF chronically in the whole brain under a neuron-specific NSE promoter, regional CBF was observed to be reduced in ischemic brain areas as a consequence of a hemodynamic steal flow (Wang et al., 2005). However, the exogenous delivery of VEGF differs from transgenic VEGF expression, as it increases regional CBF in ischemic brain tissue (Zechariah et al., 2011). Importantly, both the VEGF-induced angiogenesis and the enhancement of blood flow were blunted in animals exhibiting hyperlipidemia, leading to failure of the cerebral energy state after focal cerebral ischemia. The lack of CBF improvements under conditions of hyperlipidemia and after therapeutically induced angiogenesis, to the best of our knowledge, has never been described before.

Besides preventing the sprouting of cerebral microvessels, hyperlipidemia abolished the enhanced coverage of endothelial cells with pericytes. Pericytes exert an important regulatory role in brain microvessels, inducing the formation of blood-brain barrier properties and controlling vascular reactivity (Winkler et al., 2011). In line with the disturbed pericyte coverage, the junctional protein N-cadherin which mediates physical interactions between endothelial cells and pericytes and which was upregulated by VEGF in normolipidemic microvessels, did not respond to VEGF in hyperlipidemic mice. An interesting aspect of our results is that VEGF treatment was able to increase new vessel formation in the mildly hyperlipidemic animals while on the other hand, was not able to increase the pericyte coverage of vessels in these animals. Additionally, the increased abundance of the junctional protein N-cadherin was lost, also in the mildly hyperlipidemic animals. That hyperlipidemia prevents the

pericyte alignment with endothelial cells is noteworthy. It provides a new, hitherto unknown mechanism for disturbances of vascular reactivity in hyperlipidemia.

Indeed, the VEGF induced preservation of the BBB integrity was abolished under conditions hyperlipidemia, which was reflected by the enhanced access of serum IgG to the brain parenchyma. It is noteworthy that increased IgG extrasavation correlated with the decrease in pericyte coverage under conditions of hyperlipidemia. As pericyte coverage is crucial for maintaining a non-leaky BBB; hyperlipidemia, by counteracting pericyte contact with endothelial cells lead to the formation of leaky, dysfunctional vessels after angiogenesis therapy.

A recent investigation has demonstrated that hyperlipidemia promotes BBB permeability after focal cerebral ischemia, thereby increasing brain edema by activation of MMP-2/9 and overactivation of RhoA (Elali A et al., 2011b). By investigating MMP-9 activity in hyperlipidemic animals, we now show that hyperlipidemia reverses the preservation of extracellular matrix integrity induced by VEGF; by increasing MMP-9 activity. Besides, MMP-9 activity in the severely hyperlipidemic animals was highly exacerbated, compared to the moderately hyperlipidemic animals, further demonstrating the role of hyperlipidemia on extracellular matrix integrity.

Infarct volumetric analysis after focal cerebral ischemia demonstrated a hitherto unknown outcome that hyperlipidemia abolished the neuroprotective effect of VEGF resulting in increased infarction volumes. It could be explained by our observation that hyperlipidemia abolishes the improvement of blood flow induced by VEGF and also decreases pericyte coverage on vessels. Hence, the inability of vessels to respond to the metabolic demands of the tissue after ischemia, promoted the progression of brain injury.

6 CONCLUSIONS AND OUTLOOKS

The reasons for the failure in translation of therapeutic strategies have been a topic of intense discussion recently. Utilisation of clinically irrelevant animal models and readouts may be attributable for these discrepancies. Translational stroke research poorly mimic comorbidities as experiments are done in young and healthy animals (Hermann and Chopp, 2012).

Experimental therapeutic angiogenic strategies often chose to look at readouts which are clinically less relevant and thereby largely ignoring the changes in perfusion which is the primary purpose of inducing angiogenesis. Our investigation on healthy animals revealed the efficacy of VEGF to induce vessel formation, resulting in increased CBF and preservation of the cerebral energy state. These data supported the concept of induced angiogenesis for hitherto healthy mice.

Approximately, half of all stroke patients are hyperlipidemic. By utilising hyperlipidemic animals we showed that hyperlipidemia reverses the angiogenic responses induced by VEGF. Translational stroke studies should be aware that hyperlipidemia profoundly alters responses of cerebral blood vessels to angiogenic growth factors. The lack of vascular growth responses following VEGF treatment questions the concept of therapeutic angiogenesis in ischemic stroke.

Our investigation utilising normolipidemic and hyperlipidemic animals has demonstrated the pitfalls that result from the use of inadequate animal models. Our results argue in favour of a paradigm shift in experimental strategies that more approximately consider vascular risk factors and thus more closely resemble clinical conditions.

7 References

Abumiya T, Lucero J, Heo JH, Tagaya M, Koziol JA, Copeland BR, del Zoppo GJ (1999) Activated microvessels express vascular endothelial growth factor and integrin alpha(v)beta3 during focal cerebral ischemia. J Cereb Blood Flow Metab 19(9):1038-50.

Abumiya T, Yokota C, Kuge Y, Minematsu K (2005) Aggravation of hemorrhagic transformation by early intraarterial infusion of low-dose vascular endothelial growth factor after transient focal cerebral ischemia in rats. Brain Res 1049(1):95-103.

Adams RH, Klein R (2000) Eph receptors and ephrin ligands: essential mediators of vascular development. Trends Cardiovasc Med 10:183–8.

Allen CM (1984) Predicting the outcome of acute stroke: a prognostic score. J Neurol Neurosurg Psychiatry 47, 475–480.

Arai K, Jin G, Navaratna D, Lo EH (2009) Brain angiogenesis in developmental and pathological processes: neurovascular injury and angiogenic recovery after stroke. FEBS J. 276(17):4644-52.

Asahara T, Kawamoto A (2004) Endothelial progenitor cells for postnatal vasculogenesis. Am J Physiol Cell Physiol 287(3): C572-9.

Autiero M, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D, Kroll J, Plaisance S, De Mol M, Bono F, Kliche S, Fellbrich G, Ballmer-Hofer K, Maglione D, Mayr-Beyrle U, Dewerchin M, Dombrowski S, Stanimirovic D, Van Hummelen P, Dehio C, Hicklin DJ, Persico G, Herbert JM, Communi D, Shibuya M, Collen D, Conway EM, Carmeliet P (2003) Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. Nat Med 9(7): 936-43.

Bagnard D, Vaillant C, Khuth ST, Dufay N, Lohrum M, Puschel AW, Belin MF, Bolz J, Thomasset N (2001) Semaphorin 3A-vascular endothelial growth factor-165 balance mediates migration and apoptosis of neural progenitor cells by the recruitment of shared receptor. J Neurosci 21(10): 3332-41.

Bagri A, Tessier-Lavigne M (2002) Neuropilins as semaphoring receptors: in vivo functions in neuronal cell migration and axon guidance. Adv Exp Med Biol 515:13–31.

Beck H, Acker T, Püschel AW, Fujisawa H, Carmeliet P, Plate KH (2002) Cell type-specific expression of neuropilins in an MCA-occlusion model in mice suggests a potential role in post-ischemic brain remodeling. J Neuropathol Exp Neurol 61(4): 339-50.

Beck H, Plate KH (2009) Angiogenesis after cerebral ischemia. Acta Neuropathol. 117(5):481-96.

Bellomo M, Adamo EB, Deodato B, Catania MA, Mannucci C, Marini H, Marciano MC, Marini R, Sapienza S, Giacca M, Caputi AP, Squadrito F, Calapai G (2003) Enhancement of expression of vascular endothelial growth factor after adeno-associated virus gene transfer is associated with improvement of brain ischemia injury in the gerbil. Pharmacol Res 48(3): 309-17.

Bliss TV, Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361:31–9.

Brogi E, Wu T, Namiki A, Isner JM (1994) Indirect angiogenic cytokines upregulate VEGF and bFGF gene expression in vascular smooth muscle cells, whereas hypoxia upregulates VEGF expression only. Circulation 90(2): 649-52.

Burggraf D, Martens HK, Dichgans M, Hamann GF (2007) rt-PA causes a dose dependent increase in the extravasation of cellular and non-cellular blood elements after focal cerebral ischemia. Brain Res. 1164:55–62.

Burri PH, Hlushchuk R, Djonov V (2004) Intussusceptive angiogenesis: its emergence, its characteristics, and its significance. Dev Dyn; 231(3):474-88.

Carmeliet P (2000) Mechanisms of angiogenesis and arteriogenesis. Nat Med 6:389–395.

Carmeliet P (2003) Blood vessels and nerves: common signals, pathways and diseases. Nat Rev Genet 4: 710-720

Carmeliet P (2004) Manipulating angiogenesis in medicine. J Intern Med 255:538–561.

Carmichael ST (2003) Gene expression changes after focal stroke, traumatic brain and spinal cord injuries. Curr Opin Neurol; 16(6):699-704.

Calvo CF, Fontaine RH, Soueid J, Tammela T, Makinen T, Alfaro-Cervello C, Bonnaud F, Miguez A, Benhaim L, Xu Y, Barallobre MJ, Moutkine I, Lyytikkä J, Tatlisumak T, Pytowski B, Zalc B, Richardson W, Kessaris N, Garcia-Verdugo JM, Alitalo K, Eichmann A, Thomas JL (2011) Vascular endothelial growth factor receptor 3 directly regulates murine neurogenesis. Genes Dev. 25(8):831-44.

Chen J, Zhang C, Jiang H, Li Y, Zhang L, Robin A, Katakowski M, Lu M, Chopp M (2005) Atorvastatin induction of VEGF and BDNF promotes brain plasticity after stroke in mice. J Cereb Blood Flow Metab 25: 281-90.

Chen J, Chopp M (2006) Neurorestorative treatment of stroke: cell and pharmacological approaches. NeuroRx; 3(4): 466-73.

Cheng T, Petraglia AL, Li Z, Thiyagarajan M, Zhong Z, Wu Z, Liu D, Maggirwar SB, Deane R, Ferna´ndez JA, LaRue B, Griffin JH, Chopp M, Zlokovic BV (2006) Activated protein C inhibits tissue plasminogen activatorinduced brain hemorrhage. Nat Med. 12:1278 –1285.

Chiba Y, Sasayama T, Miyake S, Koyama J, Kondoh T, Hosoda K, Kohmura E (2008) Anti-VEGF receptor antagonist (VGA1155) reduces infarction in rat permanent focal brain ischemia. Kobe J Med Sci 54:E136-146.

Chi OZ, Hunter C, Liu X, Weiss HR (2007) Effects of anti-VEGF antibody on blood-brain barrier disruption in focal cerebral ischemia. Exp Neurol 204(1):283-7.

Choi JS, Kim HY, Cha JH, Choi JY, Park SI, Jeong CH, Jeun SS, Lee MY (2007) Upregulation of vascular endothelial growth factor receptors Flt-1 and Flk-1 following acute spinal cord contusion in rats. J Histochem Cytochem 55(8):821-30.

Chopp M, Li Y, Zhang J (2008) Plasticity and remodeling of brain. J Neurol Sci 265: 97-101.

Chopp M, Zhang ZG, Jiang Q (2007) Neurogenesis, angiogenesis, and MRI indices of functional recovery from stroke. Stroke. 38(2 Suppl):827-31.

Chu K, Park KI, Lee ST, Jung KH, Ko SY, Kang L, Sinn DI, Lee YS, Kim SU, Kim M, Roh JK (2005) Combined treatment of vascular endothelial growth factor and human neural stem cells in experimental focal cerebral ischemia. Neurosci Res 53(4): 384-90.

Croll SD, Wiegand SJ (2001) Vascular growth factors in cerebral ischemia. Mol Neurobiol 23(2-3): 121-35.

del Zoppo GJ, Mabuchi T (2003) Cerebral microvessel responses to focal ischemia. J Cereb Blood Flow Metab 23(8): 879-94.

Dickson BJ (2002) Molecular mechanisms of axon guidance. Science 298:1959–64.

Dikov MM, Ohm JE, Ray N, Tchekneva EE, Burlison J, Moghanaki D, Nadaf S, Carbone DP (2005) Differential roles of vascular endothelial growth factor receptors 1 and 2 in dendritic cell differentiation. J Immunol 174(1): 215-22.

Distler JH, Hirth A, Kurowska-Stolarska M, Gay RE, Gay S, Distler O (2003) Angiogenic and angiostatic factors in the molecular control of angiogenesis. Q J Nucl Med 47(3): 149-61.

Dixelius J, Makinen T, Wirzenius M, Karkkainen MJ, Wernstedt C, Alitalo K, Claesson-Welsh L (2003) Ligand-induced vascular endothelial growth factor receptor-3 (VEGFR-3) heterodimerization with VEGFR-2 in primary lymphatic endothelial cells regulates tyrosine phosphorylation sites. J Biol Chem 278(42): 40973-9.

Dore-Duffy P, LaManna JC (2007) Physiologic angiodynamics in the brain. Antioxid Redox Signal 9(9): 1363-71.

Duan J, Murohara T, Ikeda H, Katoh A, Shintani S, Sasaki K, Kawata H, Yamamoto N, Imaizumi T (2000) Hypercholesterolemia inhibits angiogenesis in response to hindlimb ischemia: nitric oxide-dependent mechanism. Circulation.102:III370-6.

Emerich DF, Silva E, Ali O, Mooney D, Bell W, Yu SJ, Kaneko Y, Borlongan C (2010) Injectable VEGF hydrogels produce near complete neurological and anatomical protection following cerebral ischemia in rats. Cell Transplant; 19(9):1063-71.

Elali A, Hermann DM (2011a) Liver X Receptor Activation Enhances Blood-Brain Barrier Integrity in the Ischemic Brain and Increases the Abundance of ATP-Binding Cassette Transporters ABCB1 and ABCC1 on Brain Capillary Cells. Brain Pathol. 22(2):175-87.

ElAli A, Doeppner TR, Zechariah A, Hermann DM (2011b) Increased blood-brain barrier permeability and brain edema after focal cerebral ischemia induced by hyperlipidemia: role of lipid peroxidation and calpain-1/2, matrix metalloproteinase-2/9, and RhoA overactivation. Stroke. 42:3238-44.

Ferrara N, Gerber HP, LeCouter J (2003) The biology of VEGF and its receptors. Nat Med 9(6): 669-76.

Fong GH, Rossant J, Gertsenstein M, Breitman ML (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 376(6535): 66-70.

Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 16(9): 4604-13.

Folkman J, Braunwald E, Fauci AS, Kasper DL (2000) Tumor angiogenesis, Harrision's Texbook of Internal Medicine, 15th Edition, McGraw-Hill, New York, 132-152.

Fujii T, Yonemitsu Y, Onimaru M, Inoue M, Hasegawa M, Kuwano H, Sueishi K (2008) VEGF function for upregulation of endogenous PlGF expression during FGF-2-mediated therapeutic angiogenesis. Atherosclerosis. 200(1):51-7.

Granger CV, Hamilton BB & Fiedler RC (1992) Discharge outcome after stroke rehabilitation. Stroke 23, 978–982.

Greenberg DA, Jin K (2005) From angiogenesis to neuropathology. Nature. 15; 438(7070):954-9.

Greenberg JI, Shields DJ, Barillas SG, Acevedo LM, Murphy E, Huang J, Scheppke L, Stockmann C, Johnson RS, Angle N, Cheresh DA (2008) A role for VEGF as a negative regulator of pericyte function and vessel maturation. Nature 456(7223): 809-13.

Guan W, Somanath PR, Kozak A, Goc A, El-Remessy AB, Ergul A, Johnson MH, Alhusban A, Soliman S, Fagan SC (2011) Vascular protection by angiotensin receptor antagonism involves differential VEGF expression in both hemispheres after experimental stroke. PLoS One. 6(9):e24551.

Hai J, Li ST, Lin Q, Pan QG, Gao F, Ding MX (2003) Vascular endothelial growth factor expression and angiogenesis induced by chronic cerebral hypoperfusion in rat brain. Neurosurgery 53(4): 963-70.

Hall H, Hubbell JA (2004) Matrix-bound sixth Ig-like domain of cell adhesion molecule L1 acts as an angiogenic factor by ligating alphavbeta3-integrin and activating VEGF-R2. Microvasc Res 68(3): 169-78.

Hanahan D (1997) Signaling vascular morphogenesis and maintenance. Science 277(5322): 48-50.

Han HS, Suk K (2005) The function and integrity of the neurovascular unit rests upon the integration of the vascular and inflammatory cell systems. Curr Neurovasc Res 2:409–23.

Hansen TM, Moss AJ, Brindle NPJ (2008) Vascular endothelial growth factor and angiopoietins in neurovascular regeneration and protection following stroke. Curr Neurovasc Res 5: 236-45.

Harrigan MR, Ennis SR, Sullivan SE, Keep RF (2003) Effects of intraventricular infusion of vascular endothelial growth factor on cerebral blood flow, edema, and infarct volume. Acta Neurochir (Wien) 145(1): 49-53.

Hawkins BT, Davis TP (2005) The blood-brain barrier/ neurovascular unit in health and disease. Pharmacol Rev 57:173–85.

Hayashi T, Abe K, Itoyama Y (1998) Reduction of ischemic damage by application of vascular endothelial growth factor in rat brain after transient ischemia. J Cereb Blood Flow Metab 18(8): 887-95.

Hayashi T, Noshita N, Sugawara T, Chan PH (2003) Temporal profile of angiogenesis and expression of related genes in the brain after ischemia. J Cereb Blood Flow Metab ; 23:166–180.

Hedman M, Hartikainen J, Syvänne M, Stjernvall J, Hedman A, Kivelä A, Vanninen E, Mussalo H, Kauppila E, Simula S, Närvänen O, Rantala A, Peuhkurinen K, Nieminen MS, Laakso M, Ylä-Herttuala S (2003) Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia: phase II results of the Kuopio Angiogenesis Trial. Circulation 107(21): 2677-83.

Helfrich I, Scheffrahn I, Bartling S, Weis J, von Felbert V, Middleton M, Kato M, Ergün S, Schadendorf D (2010) Resistance to antiangiogenic therapy is directed by vascular phenotype, vessel stabilization, and maturation in malignant melanoma. J Exp Med. 207:491-503.

Henry TD, Annex BH, McKendall GR, Azrin MA, Lopez JJ, Giordano FJ, Shah PK, Willerson JT, Benza RL, Berman DS, Gibson CM, Bajamonde A, Rundle AC, Fine J, McCluskey ER; VIVA Investigators (2003) The VIVA trial: Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis. Circulation. 107(10):1359-65.

Hermann DM, Kilic E, Hata R, Hossmann KA, Mies G (2001) Relationship between metabolic dysfunctions, gene responses and delayed cell death after mild focal cerebral ischemia in mice. Neuroscience. 104:947-55.

Hermann DM, Chopp M (2012) Promoting brain remodeling and plasticity for stroke recovery: Therapeutic potential, caveats and consequences for clinical translation. Lancet.11:369-80.

Hermann DM, Zechariah A (2009) Implications of vascular endothelial growth factor for postischemic neurovascular remodeling. J Cereb Blood Flow Metab. 29:1620-43.

Herz J, Reitmeir R, Hagen SI, Reinboth BS, Guo Z, Zechariah A, Elali A, Doeppner TR, Bacigaluppi M, Pluchino S, Kilic U, Kilic E, Hermann DM (2012) Intracerebroventricularly delivered VEGF promotes contralesional corticorubral plasticity after focal cerebral ischemia via mechanisms involving anti-inflammatory actions. Neurobiol Dis 45:1077-1085.

Hendel RC, Henry TD, Rocha-Singh K, Isner JM, Kereiakes DJ, Giordano FJ, Simons M, Bonow RO (2000) Effect of intracoronary recombinant human vascular endothelial growth factor on myocardial reperfusion: evidence for a dose-dependent effect. Circulation 101: 118–21.

Hennigan A, O'Callaghan RM, Kelly AM (2007) Neurotrophins and their receptors: roles in plasticity, neurodegeneration and neuroprotection. Biochem Soc Trans 35(Pt 2):424–7.

He ZJ, Huang ZT, Chen XT, Zou ZJ (2009) Effects of matrix metalloproteinase 9 inhibition on the blood brain barrier and inflammation in rats following cardiopulmonary resuscitation. Chin Med J (Engl). 122(19):2346-51.

He Z, Tessier-Lavigne M (1997) Neuropilin is a receptor for the axonal chemorepellent semaphorin III. Cell 90:739–51.

Horenstein RB, Smith DE, Mosca L (2002) Cholesterol predicts stroke mortality in the Women's Pooling Project. Stroke. 33:1863-8.

Hossmann, K.A (2006) Pathophysiology and therapy of experimental stroke. Cell. Mol. Neurobiol. 26; 1057–1083.

Hou ST, Keklikian A, Slinn J, O'Hare M, Jiang SX, Aylsworth A (2008) Sustained up-regulation of semaphorin 3A, Neuropilin1, and doublecortin expression in ischemic mouse brain during long-term recovery. Biochem Biophys Res Commun 367(1): 109-15.

Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N (1992) Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. J Biol Chem 267(36): 26031-7.

Isner JM, Pieczek A, Schainfeld R, Blair R, Haley L, Asahara T, Rosenfield K, Razvi S, Walsh K, Symes JF (1996) Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. Lancet 348(9024): 370-4.

Iso H, Jacobs DR Jr, Wentworth D, Neaton JD, Cohen JD (1989) Serum cholesterol levels and six-year mortality from stroke in 350,977 men screened for the multiple risk factor intervention trial. N Engl J Med. 320:904-10.

Jain RK, Munn LL (2000) Leaky vessels? Call Ang1!. Nat Med 6:131–2.

Jang JJ, Ho HK, Kwan HH, Fajardo LF, Cooke JP (2000) Angiogenesis is impaired by hypercholesterolemia: role of asymmetric dimethylarginine. Circulation. 102:1414-9.

Jin KL, Mao XO, Greenberg DA (2000) Vascular endothelial growth factor: direct neuroprotective effect in in vitro ischemia. Proc Natl Acad Sci USA 97(18): 10242-7.

Jones N, Iljin K, Dumont DJ, Alitalo K (2001) Tie receptors: new modulators of angiogenic and lymphangiogenic responses. Nat Rev Mol Cell Biol 2(4): 257-67.

Johanna GV, Fredy CA, David VC, Natalia MV, Angel CR, Patricia CG (2010) Rac1 activity changes are associated with neuronal pathology and spatial memory long-term recovery after global cerebral ischemia. Neurochem Int. 57(7):762-73.

Karkkainen MJ, Mäkinen T, Alitalo K (2002) Lymphatic endothelium: a new frontier of metastasis research. Nat Cell Biol 4(1):E2-5.

Kastrup J, Jørgensen E, Rück A, Tägil K, Glogar D, Ruzyllo W, Bøtker HE, Dudek D, Drvota V, Hesse B, Thuesen L, Blomberg P, Gyöngyösi M, Sylvén C; Euroinject One Group (2005) Direct intramyocardial plasmid vascular endothelial growth factor-A165 gene therapy in patients with stable severe angina pectoris A randomized double-blind placebo-controlled study: the Euroinject One trial. J Am Coll Cardiol. 5;45(7): 982-8.

Kawamura H, Li X, Goishi K, van Meeteren LA, Jakobsson L, Cébe-Suarez S, Shimizu A, Edholm D, Ballmer-Hofer K, Kjellén L, Klagsbrun M, Claesson-Welsh L (2008) Neuropilin-1 in regulation of VEGF-induced activation of p38MAPK and endothelial cell organization. Blood 112(9): 3638-49.

Kaya D, Gürsoy-Ozdemir Y, Yemisci M, Tuncer N, Aktan S, Dalkara T (2005) VEGF protects brain against focal ischemia without increasing blood--brain permeability when administered intracerebroventricularly. J Cereb Blood Flow Metab 25: 1111-8.

Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT (1989) Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 246(4935):1309-1312.

Keogh CL, Yu SP, Wei L (2007) The effect of recombinant human erythropoietin on neurovasculature repair after focal ischemic stroke in neonatal rats. J Pharmacol Exp Ther 322(2): 521-8.

Kilic E, Kilic U, Wang Y, Bassetti CL, Marti HH, Hermann DM (2006a) The phosphatidylinositol-3 kinase/Akt pathway mediates VEGF's neuroprotective activity and induces blood brain barrier permeability after focal cerebral ischemia. FASEB J 20: 1185-7.

Kilic U, Bassetti CL, Kilic E, Xing H, Wang Z, Hermann DM (2005) Post-ischemic delivery of the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor rosuvastatin protects against focal cerebral ischemia in mice via inhibition of extracellular-regulated kinase-1/-2. Neuroscience 134(3): 901-6.

Kilic U, Kilic E, Järve A, Guo Z, Spudich A, Bieber K, Barzena U, Bassetti CL, Marti HH, Hermann DM (2006b) Human vascular endothelial growth factor protects axotomized retinal ganglion cells in vivo by activating ERK-1/2 and Akt pathways. J Neurosci. 26:12439-46.

Kimura R, Nakase H, Tamaki R, Sakaki T (2005) Vascular endothelial growth factor antagonist reduces brain edema formation and venous infarction. Stroke 36(6): 1259-63.Leavesley DI, Schwartz MA, Rosenfeld M, Cheresh DA (1993) Integrin beta 1- and beta 3-mediated endothelial cell migration is triggered through distinct signaling mechanisms. J Cell Biol 121(1): 163-70.

Kogure K, Alonso OF (1978) A pictorial representation of endogenous brain ATP by a bioluminescent method. Brain Res. 154:273-84.

Koyama J, Miyake S, Sasayama T, Chiba Y, Kondoh T, Kohmura E (2010) The novel VEGF receptor antagonist, VGA1155, reduces edema, decreases infarct and improves neurological function after stroke in rats. Kobe J Med Sci 56:E1-11.

Krupinski J, Kaluza J, Kumar P, Kumar S, Wang JM (1993) Some remarks on the growth-rate and angiogenesis of microvessels in ischemic stroke. Morphometric and immunocytochemical studies. Patol Pol 44, 203–209.

Krupinski J, Kaluza J, Kumar P, Kumar S, Wang JM (1994) Role of angiogenesis in patients with cerebral ischemic stroke. Stroke 25:1794–1798.

Kukuła K, Chojnowska L, Dąbrowski M, Witkowski A, Chmielak Z, Skwarek M, Kądziela J, Teresińska A, Małecki M, Janik P, Lewandowski Z, Kłopotowski M, Wnuk J, Rużyłło W (2011) Intramyocardial plasmid-encoding human vascular endothelial growth factor A165/basic fibroblast growth factor therapy using percutaneous transcatheter approach in patients with refractory coronary artery disease (VIF-CAD). Am Heart J.161(3):581-9.

Leavesley DI, Schwartz MA, Rosenfeld M, Cheresh DA (1993) Integrin beta 1- and beta 3-mediated endothelial cell migration is triggered through distinct signalling mechanisms. J Cell Biol 121:163–70.

Lennmyr F, Ata KA, Funa K, Olsson Y, Terént A (1998) Expression of vascular endothelial growth factor (VEGF) and its receptors (Flt-1 and Flk-1) following permanent and transient occlusion of the middle cerebral artery in the rat. J Neuropathol Exp Neurol 57(9): 874-82.

Lennmyr F, Terént A, Syvänen AC, Barbany G (2005) Vascular endothelial growth factor gene expression in middle cerebral artery occlusion in the rat. Acta Anaesthesiol Scand 49(4): 488-93.

Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, Schisterman EF, Thadhani R, Sachs BP, Epstein FH, Sibai BM, Sukhatme VP, Karumanchi SA (2004) Circulating angiogenic factors and the risk of preeclampsia. N Engl J Med 350(7): 672-83.

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246(4935):1306-1309.

Lin TN, Wang CK, Cheung WM, Hsu CY (2000) Induction of angiopoietin and Tie receptor mRNA expression after cerebral ischemia-reperfusion. J Cereb Blood Flow Metab 20(2): 387-95.

Lin TN, Nian GM, Chen SF, Cheung WM, Chang C, Lin WC, Hsu CY (2001) Induction of Tie-1 and Tie-2 receptor protein expression after cerebral ischemia-reperfusion. J Cereb Blood Flow Metab 21(6): 690-701.

Liu Y, Lu JB, Ye ZR (2006) Permeability of injured blood brain barrier for exogenous bFGF and protection mechanism of bFGF in rat brain ischemia. Neuropathology 26(3): 257-66.

Li SF, Sun YB, Meng QH, Li SR, Yao WC, Hu GJ, Li ZJ, Wang RZ (2009) Recombinant adeno-associated virus serotype 1-vascular endothelial growth factor promotes neurogenesis and neuromigration in the subventricular zone and rescues neuronal function in ischemic rats. Neurosurgery 65(4):771-779; discussion 779.

Li Y, Zhang F, Nagai N, Tang Z, Zhang S, Scotney P, Lennartsson J, Zhu C, Qu Y, Fang C, Hua J, Matsuo O, Fong GH, Ding H, Cao Y, Becker KG, Nash A, Heldin CH, Li X (2008) VEGF-B inhibits apoptosis via VEGFR-1-mediated suppression of the expression of BH3-only protein genes in mice and rats. J Clin Invest ;118(3):913-23.

Li Y, Lu Z, Keogh CL, Yu SP, Wei L (2007) Erythropoietin induced neurovascular protection, angiogenesis, and cerebral blood flow restoration after focal ischemia in mice. J Cereb Blood Flow Metab 27:1043–54.

Liu Z, Li Y, Qu R, Shen L, Gao Q, Zhang X, Lu M, Savant-Bhonsale S, Borneman J, Chopp M (2007) Axonal sprouting into the denervated spinal cord and synaptic and postsynaptic protein expression in the spinal cord after transplantation of bone marrow stromal cell in stroke rats. Brain Res 1149: 172-80.

Lok J, Gupta P, Guo S, Kim WJ, Whalen MJ, van Leyen K, Lo EH (2007) Cell-cell signaling in the neurovascular unit. Neurochem Res 32:2032–45.

Losordo DW, Dimmeler S (2004) Therapeutic angiogenesis and vasculogenesis for ischemic disease. Part I: angiogenic cytokines. Circulation 109(21): 2487-91.

Maeda K, Mies G, Olah L, Hossmann KA (2000) Quantitative measurement of local cerebral blood flow in the anesthetized mouse using intraperitoneal [14C] iodoantipyrine injection and final arterial heart blood sampling. J Cereb Blood Flow Metab. 20:10-4.

Mahabeleshwar GH, Feng W, Reddy K, Plow EF, Byzova TV (2007) Mechanisms of integrin-vascular endothelial growth factor receptor cross-activation in angiogenesis. Circ Res 101(6): 570-80.

Matter CM, Ma L, von Lukowicz T, Meier P, Lohmann C, Zhang D, Kilic U, Hofmann E, Ha SW, Hersberger M, Hermann DM, Lüscher TF (2006) Increased balloon-induced inflammation, proliferation, and neointima formation in apolipoprotein E (ApoE) knockout mice. Stroke. 37(10):2625-32.

Mäkinen K, Manninen H, Hedman M, Matsi P, Mussalo H, Alhava E, Ylä-Herttuala S (2002) Increased vascularity detected by digital subtraction angiography after VEGF gene transfer to human lower limb artery: a randomized, placebo-controlled, double-blinded phase II study. Mol Ther 6(1): 127-33.

Manoonkitiwongsa PS, Jackson-Friedman C, McMillan PJ, Schultz RL, Lyden PD (2001) Angiogenesis after stroke is correlated with increased numbers of macrophages: the clean-up hypothesis. J Cereb Blood Flow Metab 21(10): 1223-31.

Manoonkitiwongsa PS, Schultz RL, McCreery DB, Whitter EF, Lyden PD (2004) Neuroprotection of ischemic brain by vascular endothelial growth factor is critically dependent on proper dosage and may be compromised by angiogenesis. J Cereb Blood Flow Metab 24(6): 693-702.

Manoonkitiwongsa PS, Schultz RL, Whitter EF, Lyden PD (2006) Contraindications of VEGF-based therapeutic angiogenesis: effects on macrophage density and histology of normal and ischemic brains. Vascul Pharmacol 44(5): 316-25.

Marti HH, Risau W (1998) Systemic hypoxia changes the organ-specific distribution of vascular endothelial growth factor and its receptors. Proc Natl Acad Sci U S A 95(26): 15809-14.

Marti HJ, Bernaudin M, Bellail A, Schoch H, Euler M, Petit E, Risau W (2000) Hypoxia-induced vascular endothelial growth factor expression precedes neovascularization after cerebral ischemia. Am J Pathol 156(3): 965-76.

Matsuzaki H, Tamatani M, Yamaguchi A, Namikawa K, Kiyama H, Vitek MP, Mitsuda N, Tohyama M (2001) Vascular endothelial growth factor rescues hippocampal neurons from glutamate-induced toxicity: signal transduction cascades. FASEB J 5(7): 1218-20.

Mies G, Ishimaru S, Xie Y, Seo K, Hossmann KA (1991) Ischemic thresholds of cerebral protein synthesis and energy state following middle cerebral artery occlusion in rat. J Cereb Blood Flow Metab 11:753-61.

Miao HQ, Soker S, Feiner L, Alonso JL, Raper JA, Klagsbrun M (1999) Neuropilin-1 mediates collapsin-1/semaphorin III inhibition of endothelial cell motility: functional competition of collapsin-1 and vascular endothelial growth factor-165. J Cell Biol 146(1): 233-42.

Miki Y, Nonoguchi N, Ikeda N, Coffin RS, Kuroiwa T, Miyatake S (2007) Vascular endothelial growth factor gene-transferred bone marrow stromal cells engineered with a herpes simplex virus type 1 vector can improve neurological deficits and reduce infarction volume in rat brain ischemia. Neurosurgery 61(3): 586-94.

Monacci WT, Merrill MJ, Oldfield EH (1993) Expression of vascular permeability factor/vascular endothelial growth factor in normal rat tissues. Am J Physiol 264(4 Pt 1): C995-1002.

Nagel S, Heinemann PV, Heiland S, Koziol J, Gardner H, Wagner S (2011) Selective MMP-inhibition with Ro 28-2653 in acute experimental stroke--a magnetic resonance imaging efficacy study. Brain Res. 1368:264-70.

Neufeld G, Kessler O, Herzog Y (2002) The interaction of neuropilin-1 and neuropilin-2 with tyrosine-kinase receptors for VEGF. Adv Exp Med Biol 515:81–90.

Ohki Y, Heissig B, Sato Y, Akiyama H, Zhu Z, Hicklin DJ, Shimada K, Ogawa H, Daida H, Hattori K, Ohsaka A (2005) Granulocyte colony-stimulating factor promotes neovascularization by releasing vascular endothelial growth factor from neutrophils. FASEB J 19(14): 2005-7.

Okada Y, Copeland BR, Hamann GF, Koziol JA, Cheresh DA, del Zoppo GJ (1996) Integrin alphavbeta3 is expressed in selected microvessels after focal cerebral ischemia. Am J Pathol 149(1): 37-44.

Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L (2006) VEGF receptor signalling - in control of vascular function. Nat Rev Mol Cell Biol 7(5): 359-71

Osto E, Matter CM, Kouroedov A, Malinski T, Bachschmid M, Camici GG, Kilic U, Stallmach T, Boren J, Iliceto S, Lüscher TF, Cosentino F (2008) c-Jun N-terminal kinase 2 deficiency protects against hypercholesterolemia-induced endothelial dysfunction and oxidative stress. Circulation. 18(20):2073-80.

Park JA, Choi KS, Kim SY, Kim KW (2003) Coordinated interaction of the vascular and nervous systems: from molecule to cell-based approaches. Biochem Biophys Res Commun 311:247–53.

Park JE, Keller GA, Ferrara N (1993) The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. Mol Biol Cell 4(12): 1317-26.

Park JE, Chen HH, Winer J, Houck KA, Ferrara N (1994) Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. J Biol Chem 269(41): 25646-54.

Park JH, Hong KS, Lee EJ, Lee J, Kim DE (2011) High levels of apolipoprotein B/AI ratio are associated with intracranial atherosclerotic stenosis. Stroke.42:3040-6.

Pepper MS, Mandriota SJ (1998) Regulation of vascular endothelial growth factor receptor-2 (Flk-1) expression in vascular endothelial cells. Exp Cell Res 241(2): 414-25.

Pinard E, Engrand N, Seylaz J (2000) Dynamic cerebral microcirculatory changes in transient forebrain ischemia in rats: involvement of type I nitric oxide synthase. J Cereb Blood Flow Metab 20(12): 1648-58.

Pitzer MR, Sortwell CE, Daley BF, McGuire SO, Marchionini D, Fleming M, Collier TJ (2003) Angiogenic and neurotrophic effects of vascular endothelial growth factor (VEGF165): studies of grafted and cultured embryonic ventral mesencephalic cells. Exp Neurol 182(2): 435-45.

Plate KH, Beck H, Danner S, Allegrini PR, Wiessner C (1999) Cell type specific upregulation of vascular endothelial growth factor in an MCA-occlusion model of cerebral infarct. J Neuropathol Exp Neurol 58(6): 654-66.

Potente M, Gerhardt H, Carmeliet P (2011) Basic and therapeutic aspects of angiogenesis. Cell.146:873-87.

Rajagopalan S, Mohler ER 3rd, Lederman RJ, Mendelsohn FO, Saucedo JF, Goldman CK, Blebea J, Macko J, Kessler PD, Rasmussen HS, Annex BH (2003) Regional angiogenesis with vascular endothelial growth factor in peripheral arterial disease: a phase II randomized, double-blind, controlled study of adenoviral delivery of vascular endothelial growth factor 121 in patients with disabling intermittent claudication. Circulation 108(16): 1933-8.

Reitmeir R, Kilic E, Kilic U, Bacigaluppi M, ElAli A, Salani G, Pluchino S, Gassmann M, Hermann DM (2011) Post-acute delivery of erythropoietin induces stroke recovery by promoting perilesional tissue remodelling and contralesional pyramidal tract plasticity. Brain. 134(Pt 1):84-99.

Reitmeir R, Kilic E, Reinboth BS, Guo Z, ElAli A, Zechariah A, Kilic U, Hermann DM (2012) Vascular endothelial growth factor induces contralesional corticobulbar plasticity and functional neurological recovery in the ischemic brain. Acta Neuropathol. 123(2):273-84.

Rivard A, Berthou-Soulie L, Principe N, Kearney M, Curry C, Branellec D, Semenza GL, Isner JM (2000) Age-dependent defect in vascular endothelial growth factor expression is associated with reduced hypoxia-inducible factor 1 activity. J Biol Chem 275(38): 29643-7.

Ripa RS, Wang Y, Jørgensen E, Johnsen HE, Hesse B, Kastrup J (2006) Intramyocardial injection of vascular endothelial growth factor-A165 plasmid followed by granulocyte-colony stimulating factor to induce angiogenesis in patients with severe chronic ischaemic heart disease. Eur Heart J. 27(15):1785-92.

Rosenstein JM, Mani N, Khaibullina A, Krum JM (2003) Neurotrophic effects of vascular endothelial growth factor on organotypic cortical explants and primary cortical neurons. J Neurosci 23(35): 11036-44.

Roskoski R Jr (2008) VEGF receptor protein-tyrosine kinases: structure and regulation. Biochem Biophys Res Commun 375(3): 287-91.

Ruhrberg C, Gerhardt H, Golding M, Watson R, Ioannidou S, Fujisawa H, Betsholtz C, Shima DT (2002) Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. Genes Dev 16(20): 2684-98.

Ruel M, Beanlands RS, Lortie M, Chan V, Camack N, deKemp RA, Suuronen EJ, Rubens FD, DaSilva JN, Sellke FW, Stewart DJ, Mesana TG (2008) Concomitant treatment with oral L-arginine improves the efficacy of surgical angiogenesis in patients with severe diffuse coronary artery disease: the Endothelial Modulation in Angiogenic Therapy randomized controlled trial. J Thorac Cardiovasc Surg.135(4):762-70.

Sacco RL, Diener HC, Yusuf S, Cotton D, Ounpuu S, Lawton WA, Palesch Y, Martin RH, Albers GW, Bath P, Bornstein N, Chan BP, Chen ST, Cunha L, Dahlöf B, De Keyser J, Donnan GA, Estol C, Gorelick P, Gu V, Hermansson K, Hilbrich L, Kaste M, Lu C, Machnig T, Pais P, Roberts R, Skvortsova V, Teal P, Toni D, Vandermaelen C, Voigt T, Weber M, Yoon BW; PRoFESS Study Group (2008) Aspirin and extended-release dipyridamole versus clopidogrel for recurrent stroke. N Engl J Med. 359(12):1238-51.

Sawada N, Salomone S, Kim HH, Kwiatkowski DJ, Liao JK (2008) Regulation of endothelial nitric oxide synthase and postnatal angiogenesis by Rac1. Circ Res.103:360-8.

Schneider A, Krüger C, Steigleder T, Weber D, Pitzer C, Laage R, Aronowski J, Maurer MH, Gassler N, Mier W, Hasselblatt M, Kollmar R, Schwab S, Sommer C, Bach A, Kuhn HG, Schäbitz WR (2005) The hematopoietic factor G-CSF is a neuronal ligand that counteracts programmed cell death and drives neurogenesis. J Clin Invest 115(8): 2083-98.

Seghezzi G, Patel S, Ren CJ, Gualandris A, Pintucci G, Robbins ES, Shapiro RL, Galloway AC, Rifkin DB, Mignatti P (1998) Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. J Cell Biol 141(7): 1659-73.

Semenza GL (2007) Vasculogenesis, angiogenesis, and arteriogenesis: mechanisms of blood vessel formation and remodeling. J Cell Biochem. 102(4):840-7.

Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219(4587):983-985.

Seylaz J, Charbonné R, Nanri K, Von Euw D, Borredon J, Kacem K, Méric P, Pinard E (1999) Dynamic in vivo measurement of erythrocyte velocity and flow in capillaries and of microvessel diameter in the rat brain by confocal laser microscopy. J Cereb Blood Flow Metab 19(8): 863-70.

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 376(6535): 62-6.

Shen F, Su H, Fan Y, Chen Y, Zhu Y, Liu W, Young WL, Yang GY (2006) Adeno-associated viral-vector-mediated hypoxia-inducible vascular endothelial growth factor gene expression attenuates ischemic brain injury after focal cerebral ischemia in mice. Stroke ;37(10):2601-6.

Shibuya M, Yamaguchi S, Yamane A, Ikeda T, Tojo A, Matsushime H, Sato M (1990) Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. Oncogene 5(4): 519-24.

Shibuya M (2006) Vascular endothelial growth factor (VEGF)-Receptor2: its biological functions, major signaling pathway, and specific ligand VEGF-E. Endothelium 13(2): 63-9.

Shimamura N, Matchett G, Yatsushige H, Calvert JW, Ohkuma H, Zhang J (2006) Inhibition of integrin alphavbeta3 ameliorates focal cerebral ischemic damage in the rat middle cerebral artery occlusion model. Stroke 37(7): 1902-9.

Shin YJ, Choi JS, Choi JY, Hou Y, Cha JH, Chun MH, Lee MY (2010) Induction of vascular endothelial growth factor receptor-3 mRNA in glial cells following focal cerebral ischemia in rats. J Neuroimmunol. 229(1-2):81-90.

Simons M, Ware JA (2003) Therapeutic angiogenesis in cardiovascular disease. Nat Rev Drug Discov. 2:863-71.

Soker S, Fidder H, Neufeld G, Klagsbrun M (1996) Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that bind VEGF165 via its exon 7-encoded domain. J Biol Chem 271(10): 5761-7.

Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M (1998) Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell 92(6): 735-45.

Stewart DJ, Hilton JD, Arnold JM, Gregoire J, Rivard A, Archer SL, Charbonneau F, Cohen E, Curtis M, Buller CE, Mendelsohn FO, Dib N, Page P, Ducas J, Plante S, Sullivan J, Macko J, Rasmussen C, Kessler PD, Rasmussen HS (2006) Angiogenic gene therapy in patients with nonrevascularizable ischemic heart disease: a phase 2 randomized, controlled trial of AdVEGF(121) (AdVEGF121) versus maximum medical treatment. Gene Ther 13(21): 1503-11.

Stowe AM, Plautz EJ, Eisner-Janowicz I, Frost SB, Barbay S, Zoubina EV, Dancause N, Taylor MD, Nudo RJ (2007) VEGF protein associates to neurons in remote regions following cortical infarct. J Cereb Blood Flow Metab 27(1): 76-85.

Sun Y, Jin K, Xie L, Childs J, Mao XO, Logvinova A, Greenberg DA (2003) VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. J Clin Invest 111: 1843–51.

Szpak GM, Lechowicz W, Lewandowska E, Bertrand E, Wierzba-Bobrowicz T & Dymecki J (1999) Border zone neovascularization in cerebral ischemic infarct. Folia Neuropathol 37, 264–268.

Tagaya M, Haring HP, Stuiver I, Wagner S, Abumiya T, Lucero J, Lee P (2001) Rapid loss of microvascular integrin expression during focal brain ischemia reflects neuron injury. J Cereb Blood Flow Metab 21(7): 835-46.

Takenaga Y, Takagi N, Murotomi K, Tanonaka K, Takeo S (2009) Inhibition of Src activity decreases tyrosine phosphorylation of occludin in brain capillaries and attenuates increase in permeability of the blood-brain barrier after transient focal cerebral ischemia. J Cereb Blood Flow Metab. 29(6): 1099-108.

Terman BI, Carrion ME, Kovacs E, Rasmussen BA, Eddy RL, Shows TB(1991) Identification of a new endothelial cell growth factor receptor tyrosine kinase. Oncogene 6(9): 1677-83.

Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD, McDonald DM (1999) Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. Science 286(5449): 2511-4.

Tille JC, Wood J, Mandriota SJ, Schnell C, Ferrari S, Mestan J, Zhu Z, Witte L, Pepper MS (2001) Vascular endothelial growth factor (VEGF) receptor-2 antagonists inhibit VEGF- and basic fibroblast growth factor-induced angiogenesis in vivo and in vitro. J Pharmacol Exp Ther 299(3): 1073-85.

Turan TN, Makki AA, Tsappidi S, Cotsonis G, Lynn MJ, Cloft HJ, Chimowitz MI; WASID Investigators (2008) Risk factors associated with severity and location of intracranial arterial stenosis. Stroke. 41(8):1636-40.

Varner JA, Brooks PC, Cheresh DA (1995) the integrin alpha V beta 3: angiogenesis and apoptosis. Cell Adhes Commun 3:367–374.

Vogel J, Gehrig M, Kuschinsky W, Marti HH (2004) Massive inborn angiogenesis in the brain scarcely raises cerebral blood flow. J Cereb Blood Flow Metab. 24:849-59.

Van Belle E, Rivard A, Chen D, Silver M, Bunting S, Ferrara N, Symes JF, Bauters C, Isner JM (1997) Hypercholesterolemia attenuates angiogenesis but does not preclude augmentation by angiogenic cytokines. Circulation. 96(8):2667-74.

van Bruggen N, Thibodeaux H, Palmer JT, Lee WP, Fu L, Cairns B, Tumas D, Gerlai R, Williams SP, van Lookeren Campagne M, Ferrara N (1999) VEGF antagonism reduces edema formation and tissue damage after ischemia/reperfusion injury in the mouse brain. J Clin Invest. 104:1613–20.

Wang L, Zhang ZG, Zhang RL, Gregg SR, Hozeska-Solgot A, LeTourneau Y, Wang Y, Chopp M (2006a) Matrix metalloproteinase 2 (MMP2) and MMP9 secreted by erythropoietin-activated endothelial cells promote neural progenitor cell migration. J Neurosci. 26(22):5996-6003.

Wang L, Chopp M, Gregg SR, Zhang RL, Teng H, Jiang A, Feng Y, Zhang ZG (2008) Neural progenitor cells treated with EPO induce angiogenesis through the production of VEGF. J Cereb Blood Flow Metab 28(7): 1361-8.

Wang T, Soker S, Atala A, Siroky MB, Azadzoi KM (2004) Alterations in angiogenic growth factors and neuronal nitric oxide synthase expression in chronic cavernosal ischemia. Int J Impot Res 16(5): 403-11.

Wang X, Lee SR, Arai K, Lee SR, Tsuji K, Rebeck GW, Lo EH (2003) Lipoprotein receptor-mediated induction of matrix metalloproteinase by tissue plasminogen activator. Nat Med. 2003;9:1313–1317.

Wang Y, Dong Y, Li R, Wang D, Tang H, Liu W, Zhang X (2006b) Transmyocardial revascularization and vascular endothelial growth factor administration enhance effect of cell transplantation for myocardial infarct repair in rats. Coron Artery Dis 17(3): 275-81.

Wang Y, Galvan V, Gorostiza O, Ataie M, Jin K, Greenberg DA (2006c) Vascular endothelial growth factor improves recovery of sensorimotor and cognitive deficits after focal cerebral ischemia in the rat. Brain Res.18; 1115(1):186-93.

Wang Y, Jin K, Mao XO, Xie L, Banwait S, Marti HH, Greenberg DA (2007a) VEGF-overexpressing transgenic mice show enhanced post-ischemic neurogenesis and neuromigration. J Neurosci Res 85(4):740-7.

Wang Y, Kilic E, Kilic U, Weber B, Bassetti CL, Marti HH, Hermann DM (2005) VEGF overexpression induces post-ischaemic neuroprotection, but facilitates haemodynamic steal phenomena. Brain 128: 52-63.

Wang YL, Hui YN, Guo B, Ma JX (2007b) Strengthening tight junctions of retinal microvascular endothelial cells by pericytes under normoxia and hypoxia involving angiopoietin-1 signal way. Eye 21(12): 1501-10.

Wang YQ, Cui HR, Yang SZ, Sun HP, Qiu MH, Feng XY, Sun FY (2009) VEGF enhance cortical newborn neurons and their neurite development in adult rat brain after cerebral ischemia. Neurochem Int 55(7):629-636.

Wang YQ, Guo X, Qiu MH, Feng XY, Sun FY (2007c) VEGF overexpression enhances striatal neurogenesis in brain of adult rat after a transient middle cerebral artery occlusion. J Neurosci Res 85(1): 73-82.

Wick A, Wick W, Waltenberger J, Weller M, Dichgans J, Schulz JB (2002) Neuroprotection by hypoxic preconditioning requires sequential activation of vascular endothelial growth factor receptor and Akt. J Neurosci 22(15): 6401-7.

Wiessner C, Bareyre FM, Allegrini PR, Mir AK, Frentzel S, Zurini M, Schnell L, Oertle T, Schwab ME (2003) Anti-Nogo-A antibody infusion 24 hours after experimental stroke improved behavioral outcome and corticospinal plasticity in normotensive and spontaneously hypertensive rats. J Cereb Blood Flow Metab 23(2): 154-65.

Winkler EA, Bell RD, Zlokovic BV (2011) Central nervous system pericytes in health and disease. Nat Neurosci.14:1398-405.

Wu HM, Huang Q, Yuan Y, Granger HJ (1996) VEGF induces NO-dependent hyperpermeability in coronary venules. Am J Physiol 271(6 Pt 2): H2735-9.

Yamashita T, Ninomiya M, Hernández Acosta P, García-Verdugo JM, Sunabori T, Sakaguchi M, Adachi K, Kojima T, Hirota Y, Kawase T, Araki N, Abe K, Okano H, Sawamoto K (2006) Subventricular zone-derived neuroblasts migrate and differentiate into mature neurons in the post-stroke adult striatum. J Neurosci. 26(24):6627-36.

Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J (2000) Vascular-specific growth factors and blood vessel formation. Nature 407(6801): 242-8.

Yang JP, Liu HJ, Liu XF (2010) VEGF promotes angiogenesis and functional recovery in stroke rats. J Invest Surg 23:149-155.

Yang JP, Liu HJ, Wang ZL, Cheng SM, Cheng X, Xu GL, Liu XF (2009) The dose-effectiveness of intranasal VEGF in treatment of experimental stroke. Neurosci Lett 461:212-216.

Yano A, Shingo T, Takeuchi A, Yasuhara T, Kobayashi K, Takahashi K, Muraoka K, Matsui T, Miyoshi Y, Hamada H, Date I (2005) Encapsulated vascular endothelial growth factor-secreting cell grafts have neuroprotective and angiogenic effects on focal cerebral ischemia. J Neurosurg 103(1): 104-14.

Yonekura H, Sakurai S, Liu X, Migita H, Wang H, Yamagishi S, Nomura M, Abedin MJ, Unoki H, Yamamoto Y, Yamamoto H (1999) Placenta growth factor and vascular endothelial growth factor B and C expression in microvascular endothelial cells and pericytes. Implication in autocrine and paracrine regulation of angiogenesis. J Biol Chem 274(49): 35172-8.

Zacharek A, Chen J, Cui X, Li A, Li Y, Roberts C, Feng Y, Gao Q, Chopp M (2007) Angiopoietin1/Tie2 and VEGF/Flk1 induced by MSC treatment amplifies angiogenesis and vascular stabilization after stroke. J Cereb Blood Flow Metab 27(10): 1684-91.

Zechariah A, ElAli A, Mies G, Hermann DM (2011) VEGF-induced angiogenesis is followed by improvements of cerebral blood flow, energy metabolism and histological injury in subsequent ischemic stroke. Eur J Neurol.18:95 (abstract).

Zechariah A, ElAli A, Hermann DM (2010) Combination of tissue-plasminogen activator with erythropoietin induces blood-brain barrier permeability, extracellular matrix disaggregation, and DNA fragmentation after focal cerebral ischemia in mice. Stroke. 41:1008-12.

Zhang Z, Chopp M (2002) Vascular endothelial growth factor and angiopoietins in focal cerebral ischemia. Trends Cardiovasc Med 12(2): 62-6.

Zhang ZG, Zhang L, Jiang Q, Zhang R, Davies K, Powers C, Bruggen N, Chopp M (2000) VEGF enhances angiogenesis and promotes blood–brain barrier leakage in the ischemic brain. J Clin Invest 106: 829–38.

Zhang ZG, Zhang L, Tsang W, Soltanian-Zadeh H, Morris D, Zhang R, Goussev A, Powers C, Yeich T, Chopp M (2002) Correlation of VEGF and angiopoietin expression with disruption of blood-brain barrier and angiogenesis after focal cerebral ischemia. J Cereb Blood Flow Metab. 22(4):379-92.

Zhang ZG, Tsang W, Zhang L, Powers C, Chopp M (2001) Up-regulation of neuropilin-1 in neovasculature after focal cerebral ischemia in the adult rat. J Cereb Blood Flow Metab 21: 541-9.

Zhao H, Bao XJ, Wang RZ, Li GL, Gao J, Ma SH, Wei JJ, Feng M, Zhao YJ, Ma WB, Yang Y, Li YN, Kong YG (2011) Postacute ischemia vascular endothelial growth factor transfer by transferrin-targeted liposomes attenuates ischemic brain injury after experimental stroke in rats. Hum Gene Ther; 22(2):207-15.

Zhao Y, Li Z, Wang R, Wei J, Li G, Zhao H (2010) Angiopoietin 1 counteracts vascular endothelial growth factor-induced blood-brain barrier permeability and alleviates ischemic injury in the early stages of transient focal cerebral ischemia in rats. Neurol Res 32:748-755.

Zhu W, Mao Y, Zhao Y, Zhou LF, Wang Y, Zhu JH, Zhu Y, Yang GY (2005a) Transplantation of vascular endothelial growth factor-transfected neural stem cells into the rat brain provides neuroprotection after transient focal cerebral ischemia. Neurosurgery. 57(2):325-33; discussion 325-33.

Zhu Y, Lee C, Shen F, Du R, Young WL, Yang GY (2005b) Angiopoietin-2 facilitates vascular endothelial growth factor-induced angiogenesis in the mature mouse brain. Stroke 36(7): 1533-7.

8 PERMISSIONS

Parts of the introduction of this thesis are published and unpublished (In press) reviews:

1. Ma Y***, Zechariah A***, Qu Y, Hermann DM (2012) Effects of vascular endothelial growth factor in ischemic stroke. **J Neurosci Res.** In press. * Equal contribution
2. Hermann DM, **Zechariah A** (2009) Implications of vascular endothelial growth factor for postischemic neurovascular remodeling. **J Cereb Blood Flow Metab**. 29(10):1620-43.
   Link: http://www.nature.com/jcbfm/journal/v29/n10/full/jcbfm2009100a.html

The results presented in this thesis are parts of manuscripts that are currently under review:

1. **Zechariah A**, Jin F, Doeppner TR, ElAli A, Schäfer ST, Helfrich I, Mies G, Hermann DM (2012) Hyperlipidemia attenuates VEGF-induced angiogenesis, impairs cerebral blood flow and disturbs stroke recovery via reduced VEGF receptor-2 and -1 expression and decreased pericyte coverage of brain endothelial cells. **Brain.** Under review.

2. **Zechariah A**, ElAli A, Doeppner TR, Jin F, Helfrich I, Mies G, Hermann DM (2012) VEGF promotes pericyte coverage of brain capillaries, improves cerebral blood flow during subsequent focal cerebral ischemia, and preserves the metabolic penumbra. **Stroke.** Under revision.

9. CURRICULUM VITAE

"The biography is not included in the online version for reasons of data protection".

10. PUBLICATIONS

1. **Zechariah A**, Jin F, Doeppner TR, ElAli A, Schäfer ST, Helfrich I, Mies G, Hermann DM (2012) Hyperlipidemia attenuates VEGF-induced angiogenesis, impairs cerebral blood flow and disturbs stroke recovery via reduced VEGF receptor-2 and -1 expression and decreased pericyte coverage of brain endothelial cells. **Brain.** Under review.

2. **Zechariah A**, ElAli A, Doeppner TR, Jin F, Helfrich I, Mies G, Hermann DM (2012) VEGF promotes pericyte coverage of brain capillaries, improves cerebral blood flow during subsequent focal cerebral ischemia, and preserves the metabolic penumbra. **Stroke.** Under revision.

3. Ma Y***, Zechariah A***, Qu Y, Hermann DM (2012) Effects of vascular endothelial growth factor in ischemic stroke. **J Neurosci Res.** Jun 20. doi: 10.1002/jnr.23088.* *Equal contribution*

4. Doeppner TR, Ewert AS.T, Tönges L, Herz J, **Zechariah A**, ElAli A, Ludwig A-K, Giebel B, Nagel F, Dietz G. PH, Weise J, Hermann DM, Bähr M (2012) Transduction of neural precursor cells with TAT-Hsp70 chaperone: therapeutic potential against ischemic stroke after intrastriatal and systemic transplantation. **Stem Cells.** In press.

5. Herz J, Reitmeir R, Hagen SI, Reinboth BS, Guo Z, **Zechariah A,** ElAli A, Doeppner TR, Bacigaluppi M, Pluchino S, Kilic U, Kilic E, Hermann DM (2012) Intracerebroventricularly delivered VEGF promotes contralesional corticorubral plasticity after focal cerebral ischemia via mechanisms involving anti-inflammatory actions. **Neurobiol Dis.** 45(3):1077-85.

6. Reitmeir R, Kilic E, Reinboth BS, Guo Z, ElAli A, **Zechariah A,** Kilic U, Hermann DM (2012) Vascular endothelial growth factor induces contralesional corticobulbar plasticity and functional neurological recovery in the ischemic brain. **Acta Neuropathol.** 123(2):273-84.

7. ElAli A, Doeppner TR, **Zechariah A**, Hermann DM (2011) Increased blood-brain barrier permeability and brain edema after focal cerebral ischemia induced by hyperlipidemia: role of lipid peroxidation and calpain-1/2, matrix metalloproteinase-2/9, and RhoA overactivation. **Stroke.** 42(11):3238-44.

8. Doeppner TR, Kaltwasser B, ElAli A, **Zechariah A**, Hermann DM, Bähr M (2011) Acute hepatocyte growth factor treatment induces long-term neuroprotection and stroke recovery via mechanisms involving neural precursor cell proliferation and differentiation**. J Cereb Blood Flow Metab**. 31(5):1251-62.

9. **Zechariah A**, ElAli A, Hermann DM (2010) Combination of tissue-plasminogen activator with erythropoietin induces blood-brain barrier permeability, extracellular matrix disaggregation, and DNA fragmentation after focal cerebral ischemia in mice. **Stroke**. 41(5):1008-12.

10. Hermann DM, **Zechariah A** (2009) Implications of vascular endothelial growth factor for postischemic neurovascular remodeling. **J Cereb Blood Flow Metab**. 29(10):1620-43.

11. Chen CC*, **Zechariah A***, Hsu YH, Chen HW, Yang LC, Chang C (2008) Neuroaxonal ion dyshomeostasis of the normal-appearing corpus callosum in experimental autoimmune encephalomyelitis**. Exp Neurol.** 210(2):322-30. * *Equal contribution*

12. Hsu YH, Chen CC, **Zechariah A**, Yen CC, Yang LC, Chang C (2008) Neuronal dysfunction of a long projecting multisynaptic pathway in response to methamphetamine using manganese-enhanced MRI. **Psychopharmacology**. 196(4):543-53.

11 ERKLÄRUNG

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, f der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „*VEGF-induced angiogenesis in the ischemic brain: Effect of hyperlipidemia*“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von (Anil Zechariah) befürworte.

Essen, den 08.08.2012

Prof Dr. Drik M.Hermann	Dirk Hermann
_____	_____
Name des wissenschaftl. Betreuers/Mitglieds der Universität Duisburg-Essen	Unterschrift d. wissenschaftl. Betreuers/ Mitglieds der Universität Duisburg-Essen

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. 2, c und e der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

Anil Zechariah

Essen, den 08.08.2012 _____

Unterschrift des/r Doktoranden/in

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. 2, d und f der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe, dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist, und dass ich die Dissertation nur in diesem Verfahren einreiche.

Anil Zechariah

Essen, den 08.08.2012 _____

Unterschrift des/r Doktoranden/in